(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 198 328 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication of patent specification: **01.07.92**

(51) Int. Cl.5: **C12N 15/86**, C12N 7/00, A61K 39/015

(21) Application number: **86104498.0**

(22) Date of filing: **02.04.86**

(54) Vaccinia DNA.

(30) Priority: **04.04.85 GB 8508845**

(43) Date of publication of application:
22.10.86 Bulletin 86/43

(45) Publication of the grant of the patent:
01.07.92 Bulletin 92/27

(84) Designated Contracting States:
BE CH DE FR GB IT LI NL SE

(56) References cited:
EP-A- 0 110 385

PROC. NATL. ACAD. SCI. USA, vol. 82, no. 7, April 1985, pages 2096-2100; C. BERTHOLET et al.: "One hundred base pairs of 5' flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription"

PROC. NATL. ACAD. SCI. USA, vol. 80, no. 17, September 1983, pages 5364-5368, Washington, US; D. PANICALI et al.: "Construction of live vaccines by using genetically engineered poxviruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin"

PROC. NATL. ACAD. SCI. USA, vol. 81, no. 1, January 1984, pages 193-197; E. PAOLETTI et al.: "Construction of live vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D"

(73) Proprietor: **F. HOFFMANN-LA ROCHE AG**
Postfach 3255
CH-4002 Basel(CH)

(72) Inventor: **Stunnenberg, Hendrik Gerard**
6 Johan Sebastian Bach Strasse
W-6901 Bammental(DE)
Inventor: **Wittek, Riccardo**
Chemin de Cocagne
CH-1030 Bussigny(CH)

(74) Representative: **Lederer, Franz, Dr. et al**
Lederer, Keller & Riederer Patentanwälte
Lucile-Grahn-Strasse 22
W-8000 München 80(DE)

EP 0 198 328 B1

B3

Description

Vaccinia virus is the prototype for the orthopoxvirus of the poxvirus family. Its biology and replication has been described extensively by B. Moss ("Poxviruses", in Comprehensive Virology, eds. H. Fraenkel-Conrat and R. Wagner, Plenum Press, New York, Vol. 4, pp. 405-474 [1974]: "Poxviruses", in Molecular Biology in Animal Viruses, ed. D.P. Nayak, Marcel Dekker, New York, Vol. 2, pp. 849-890 [1978]; "5' end labelling of RNA with capping and methylating enzymes", in Gene Amplification and Analysis, eds. J. G. Chirikjian and T. S. Papas, Elsevier, North-Holland, Vol. 2, pp. 254-266 [1981]; "Principles of virus replication: poxvirus", in Human Viral Diseases, eds. B. N. Fields, R. Chanock, R. Shope and B. Roizman, Raven Press, New York, in press [1984]). While several types of animal DNA viruses with large genomes have been used as cloning vectors, including adenovirus, herpes simplex virus and vaccinia virus only recombinants of the latter have expressed foreign genes while retaining complete infectivity. Meanwhile great experience has been gained with the use of vaccinia virus as a live vaccine. Its wide host range, large capacity for foreign DNA and inability to induce oncogenic transformation are all features enhancing the potential of vaccinia virus recombinants as live vaccines. An updated review of the use of recombinant vaccinia viruses as live vaccines has been given by G. L. Smith et al. (Biotechnology and Genetic Engineering Reviews 2, 383-407, [1984]), including a description of the biology of recombinant vaccinia viruses and the expression of foreign genes under the control of vaccinia promoters.

The present invention deals with vaccinia DNA preferably transcriptional regulatory sequences from the 5' flanking region of the vaccinia virus late gene encoding a basic polypeptide with a molecular weight of 11000 (11 kDa), recombination vectors useful for the insertion of foreign genes into poxvirus, recombinant infectious poxviruses containing foreign genes operatively linked to such transcriptional regulatory sequences and capable of effecting expression of the corresponding polypeptide and live vaccines on the basis of such recombinant infectious poxviruses. Preferred poxviruses, used in this invention are vaccinia viruses.

The gene coding for a major late 11 kDa structural polypeptide of the vaccinia virus which has been mapped by R. Wittek et al. (J. Virol. 49, 371-378 [1984]) has been sequences including its 5'-flanking region. The DNA sequence of above gene and derived amino acid sequence is indicated below:

```
GTACCAAATTCTTCTATCTCTTTAACTACTTGCATAGATAGGTAATTACAGTGATGCCTAC

ATGCCGTTTTTTGAAACTGAATAGATGCGTCTAGAAGCGATGCTACGCTAGTCACAATCAC

CACTTTCATATTTAGAATATATGTATGTAAAAATATAGTAGAATTTCATTTTGTTTTTTTC

          MetAsnSerHisPheAlaSerAlaHisThrProPheTyrIleAsnThrLys
TATGCTATAAATGAATTCTCATTTTGCATCTGCTCATACTCCGTTTTATATCAATACCAAA

GluGlyArgTyrLeuValLeuLysAlaValLysValCysAspValArgThrValGluCys
GAAGGAAGATATCTGGTTCTAAAAGCCGTTAAAGTATGCGATGTTAGAACTGTAGAATGC

GluGlySerLysAlaSerCysValLeuLysValAspLysProSerSerProAlaCysGlu
GAAGGAAGTAAAGCTTCCTGCGTACTCAAAGTAGATAAACCCTCATCGCCCGCGTGTGAG

ArgArgProSerSerProSerArgCysGluArgMetAsnAsnProArgLysGlnValPro
AGAAGACCTTCGTCCCCGTCCAGATGCGAGAGAATGAATAACCCTAGAAAACAAGTTCCG

PheMetArgThrAspMetLeuGlnAsnMetPheAlaAlaAsnArgAspAsnValAlaSer
TTTATGAGGACGGACATGCTACAAAATATGTTCGCGGCTAATCGCGACAACGTGGCGTCG

ArgLeuLeuAsn
AGGCTTTTGAACTAAAATACAATTATATCCTTTTCGATATTAATAAATCCGTGTCGTCAA

GGTTTTTTATC
```

The 5'-flanking region shows little homology to either the corresponding region of vaccinia early genes (Weir, J. P. and B. Moss, J. Virol. 51, 662-669 [1984]) or to consensus sequences characteristic of most

eukaryotic genes. Furthermore, it has been found that a DNA fragment of not more than about 100 base pairs from the 5'-flanking region of the 11 kDa gene contains all necessary transcriptional regulatory signals for correct regulation of vaccinia virus late gene expression.

Therefore, the present invention comprises a transcriptional regulatory sequence of the following formula

```
                                    100
 5'        CTAGA          AGCGA    TGCTA

90                         80
 CGCTA     GTCAC          AATCA    CCACT

70                         60
 TTCAT     ATTTA          GAATA    TATGT

50                         40
 ATGTA     AAAAT          ATAGT    AGAAT

30                         20
 TTCAT     TTTGT          TTTTT    TCTAT

10
 GCTAT     AAATG          3'
```

or subunits (fragments) thereof which are capable of regulating expression of foreign genes.

In addition it has been found that a DNA fragment of not more than 13 base pairs counted from position 2 at the 3'-end of above transcriptional regulatory sequence constitutes a preferred element for correct regulation of vaccinia virus late gene expression. Therefore such 3'-end transcriptional regulatory sequences (fragments) also belong to the present invention and are encompassed by the present application.

Furthermore, the invention comprises variations of any of the foregoing transcriptional regulatory sequences including deletions, insertions, substitutions, inversions of single or several nucleotides and combinations thereof capable of functioning as a poxvirus late promoter (functional variations).

Specific examples of functional variations of the above mentioned transcriptional regulatory sequences are represented by the following formulas:

```
5'      CTAGA    AGCGA    TGCTA

  CGCTA    GTCAC    AATCA    CCACT

  TTCAT    ATTTA    GAATA    TATGT

  ATGTA    AAAAT    ATAGT    AGAAT

  TTCAT    TTTGT    TTTTT    AAAGG

  ATCTA    TAAAT    AAAT     3'
```

and

```
5'      CTAGA    AGCGA    TGCTA

  CGCTA    GTCAC    AATCA    CCACT

  TTCAT    ATTTA    GAATA    TATGT

  ATGTA    AAAAT    ATAGT    AGAAT

  TTCAT    TTTGT    TTTTT    TCTAT

  CGATT    AAATA    AAG      3'
```

The invention also encompasses tandem repeats, e.g. from 2 to 5 times, of the above mentioned transcriptional regulatory sequences.

The invention further comprises recombination vectors containing a chimeric gene consisting of at least one transcriptional regulatory sequence of the vaccinia major late 11 kDa gene operatively linked to a foreign gene encoding prokaryotic or eukaryotic polypeptides, DNA from a non-essential segment of the poxvirus genome flanking said chimeric gene, the vector origin of replication and antibiotic resistence genes. The translational initiation site of the chimeric gene is provided either by the transcriptional regulatory sequence of the vaccinia major 11 kDa gene or by the foreign gene encoding prokaryotic or eukaryotic polypeptides. By using the translational initiation site of the foreign gene codon phasing and potential problems associated with biological activity of fusion proteins are avoided. Preferred recombination vectors using the translational initiation site of the transcriptional regulatory sequence of the vaccinia major 11 kDa gene or the translational initiation site of the foreign gene are described in the examples 1-4.

The recombination vectors of this invention can be constructed by methods well known in the art (D. Panicali and E. Paoletti, PNAS 79, 4927-4931 [1983]; D. Panicali et al., PNAS 80, 5364-5368 [1984]; G. L. Smith et al., supra; M. Mackett et al., J. Virol. 49, 857-864 [1984]) comprising the steps of:

(a) preparing a vector containing poxvirus DNA, said DNA comprising:

(i) at least one transcriptional regulatory sequence next to at least one restriction endonuclease site, and

(ii) DNA from a non-essential segment of the poxvirus genome flanking said regulatory sequence and said restriction endonuclease site; and

(b) inserting at least one foreign gene encoding prokaryotic or eukaryotic polypeptides into said restriction endonuclease site next to said transcriptional regulatory sequence.

Intermediate recombination vectors comprising at least one transcriptional regulatory sequence of the vaccinia major late 11 kDa gene and still lacking a foreign gene encoding prokaryotic or eukaryotic polypeptides are also an object of the present invention and can be prepared by the above step (a) wherein the transcriptional regulatory sequence of the vaccinia major late 11 kDa gene includes the translational inition site of the 11 kDa gene or, optionally, is terminated in the region between the mRNA start and the translational inition site of the 11 kDa gene.

The vector used to assemble the recombination vector may be any convenient plasmid, cosmid, or phage. Convenient vehicles of plasmid, cosmid or phase origin are mentioned e.g., in the laboratory manual "Molecular Cloning" by Maniatis et al., Cold Spring Harbor laboratory, 1982. Preferred vectors of plasmid origin, used to assemble the recombination vectors in this invention are pBR322 and pUC8.

The DNA used to flank the chimeric gene may be derived from non-essential regions of the poxvirus genome. Examples of such non-essential regions include the thymidine kinase (TK) gene (J. P. Weir and B. Moss, J. Virol. 46, 530-537 [1983]). The preferred non-essential regions used in this invention comprise a segment of the poxvirus thymidine kinase gene and DNA adjacent to said thymidine kinase gene. Especially preferred is a segment of the vaccinia virus thymidine kinase gene and vaccinia DNA adjacent to said vaccinia thymidine kinase gene. the preparation of the non-essential regions is described more in detail in example 1.

Foreign genes that may be inserted into the recombination vectors of this invention may be selected from a large variety of genes (DNA genes or DNA copies of RNA genes) that encode prokaryotic or eukaryotic polypeptides. For example, such genes may encode enzymes, hormones, polypeptides with immuno-modulatory, anti-viral or anti-cancer properties, antibodies, antigens, and other useful polypeptides of prokaryotic or eukaryotic origin. Preferred foreign genes used in this invention are the genes encoding malaria antigens, in particular the 5.1 surface antigen of Plasmodium falciparum (Hope, I.A. et al., infra) and the mouse dihydrofolate reductase (DHFR) gene (A.C.Y. Chang et al., "Phenotypic expression in E. coli of a DNA sequence coding for mouse dihydrofolate reductase", Nature 275, 617-624 [1978]).

Plasmids of the pHGS family are specific examples of plasmid recombination vectors of the present invention. Their preparation is described more in detail in examples 1-4. E. coli strains containing plasmids useful for the preparation of the recombination vectors of the present invention (E. coli HB 101 transformed with pHGS-1; pHGS-2) were deposited at Deutsche Sammlung von Mikroorganismen (DSM) in Göttingen on February 21, 1985 the accession nos. being DSM 3248 and DSM 3249 respectively. Other gram-negative hosts such as E. coli C 600 (E. coli DH 1) and E. coli RR1 (ATCC No. 31343) can also be used and are described in the laboratory manual "Molecular Cloning" by Maniatis et al., supra.

Suitable recombinant infectious poxviruses containing and expressing the above mentioned chimeric genes can be obtained by methods well known in the art (G.L. Smith et al., Biotechnology and Genetic Engeneering Reviews 2, 383-407 [1984]; M. Mackett et al., supra) comprising the steps of:

(a) providing at least one cell infected with a genus of poxvirus;

(b) transfecting said cell with a recombination vector, whereby homologous recombination occurs between the DNA of the poxvirus and at least one portion of the poxvirus DNA contained in the recombination vector; and

(c) isolating from said cell a recombinant infectious poxvirus capable of expressing said foreign gene encoding prokaryotic or eukaryotic polypeptides by selective methods.

Suitable eukaryotic host organisms, which can be used for the manufacture of a recombinant infectious poxvirus include CV-1, RK-13, TK$^-$143, or other cells. The preferred eukaryotic host cell used in this invention is RK-13.

Recombinant infectious vaccinia viruses RVV 1 through 8 are specific examples of the present invention. Details of their preparation and isolation are indicated in examples 1-4.

Examples of proteins which can be expressed by using the recombinant infectious vaccinia viruses of the present invention are mouse dihydrofolate reductase, chloramphenicol acetyltransferase and malaria surface antigens, in particular the 5.1 surface antigen of Plasmodium falciparum.

Methods for expressing chimeric genes encoding prokaryotic or eukaryotic polypeptides using recombinant infectious poxviruses are well known. (G.L. Smith et al., supra; M.P. Kieny et al., Nature 312, 163-166 [1984]; G.L. Smith et al., Science 224, 397-399 [1984]; E. Paoletti et al., PNAS 81, 193-197 [1984]; D. Panicali et al., supra). They include infecting an appropriate host with a recombinant infectious poxvirus having the desired foreign gene operatively linked to the poxvirus transcriptional regulatory sequence, incubating the host under appropriate conditions and detecting the desired polypeptide by immunological, enzymatic, and electrophoretic methods.

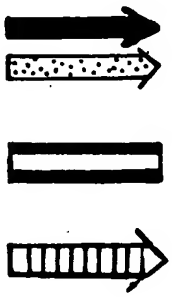
The recombinant infectious poxviruses can therefore be used as live vaccines by inoculating an animal or human with an inoculant containing a concentration of said recombinant infectious poxvirus sufficient to

elicit an immunological response in said animal or human comprising the production of antibodies to at least the antigenic portion of the protein encoded by said foreign gene. Preferred recombinant infectious poxviruses used in this invention for protective immunization are recombinant infectious vaccinia viruses. Especially preferred are those expressing malaria surface antigens, in particular the 5.1 surface antigen of Plasmodium falciparum.

Recombinant infectious vaccinia viruses for use in man can be prepared as described by C. Kaplan (Br. med. Bull. 25, 131-135 [1969]). Preparations suitable for vaccination must contain 10^5 to 10^8 plaque forming units per 0.05 ml. The vaccine can be stored frozen in aqueous buffer solution containing 40-50% glycerol, or in lyophilized form. This lyophilized form of the vaccine is essential for use in underdeveloped areas. Vaccination is achieved as a result of intradermal inoculation. A drop of the vaccine is applied to a small sterlized area of the skin and the epidermis beneath is then rapidly punctured by means of a sharp sterile needle or Knife. In mass vaccination compaigns jet guns are used.

Having now generally described this invention, the same will be better understood on the basis of the following examples when considered in connection with the following figures:

The symbols used are:



representing the vaccinia virus TK-gene;

representing the transcriptional regulatory sequences of the 11kDa protein of vaccinia virus;

representing the gene for the 5.1 Antigen of Plasmodium falciparum.

representing the gene for mouse dihydrofolate reductase.

Figure 1 Part a) is a schematic outline of the construction of plasmid pBR-J consisting of pBR-322 and the Hind III "J" fragment of vaccinia virus, containing the vaccinia virus TK-gene.
Part b) is a schematic outline of the construction of plasmid pBR-F1 consisting of pBR-322 and the righthand side of the Hind III-F fragment of vaccinia virus, containing the transcriptional regulatory sequences of the 11kDa protein of vaccinia virus.

Figure 2 is a schematic outline of the subcloning of the vaccinia virus TK-gene plus flanking sequences into plasmid pUC-8 resulting in plasmid pUC-TK.

Figure 3 is a schematic outline of the insertion of the transcriptional regulatory sequences of the 11kDa protein of vaccinia virus into the TK-gene resulting in plasmid pUC-TK/11kDa.

Figure 4 is a schematic outline of the conversion of the upstream EcoRI restriction endonuclease site of the transcriptional regulatory sequences of the 11kDa protein into an Xmn I restriction endonuclease site resulting in plasmid pHGS-1.

Figure 5 Part a) is a schematic outline of the isolation and radioactive labelling of the nuclease S1 probe for transcripts starting at the transcriptional regulatory sequences of the 11kDa protein inserted into the TK-gene.
Part b) is a schematic outline of the isolation and radioactive labelling of the nuclease S1 probe for transcripts starting at the regulatory sequences of the TK-gene.

Figure 6 The upper part is an outline of mapping of RNA transcripts (⇝) with nuclease S1.
The lower part is the x-ray exposure of the nuclease S1 mapping of TK transcript (250 bp) and 11kDa transcript (260 bp). RNA from infected cells were harvested at 3 hours (lane 1 and 2) resp. 7 hours (lane 3 and 4) post infection.

Figure 7 The upper part represents an outline of the mapping of RNA transcripts (⇝) with nuclease S1. The lower part is the x-ray exposure of the nuclease S1 mapping of RNA transcripts from cells infected with RVV-2 (pHGS-2, lane 1 and 2) resp. RVV-3 (pHGS-2Δ15, lane 3 and 4). RNA was extracted at 4 hours (lane 1 and 4) resp. 8 hours (lane 2 and 3) post infection. Lanes marked "M" consist of ^{32}P-labelled Hpa II fragments of pBR-322 giving the length position (in bp) as indicated. The position of the S1 protected band corresponding to RNA transcripts starting at the regulatory sequences of the TK-gene resp. the inserted 11kDa are indicated.

Figure 8 represents a schematic outline of the cloning of the Plasmodium falciparum 5.1 Antigen

(Hope et al. supra) into the plasmid pHGS-2 resulting in the plasmid pHGS-2/5.1.

Figure 9 represents indirect immunofluorescence of cells infected with the virus RVV-4 containing the Plasmodium falciparum 5.1 Antigen. Panel A cells photographed at 450-490 nm. panel B with phase contrast.

Figure 10 represents a schematic outline of the construction of plasmid pHGS-2/DHFR.

Figure 11 represents a schematic outline of the construction of plasmid pHGS-2/DHFR-E.

Figure 12 represents a schematic outline of the construction of plasmid pHGS-A/DHFR.

Figure 13 represents a schematic outline of the construction of plasmid pHGS-F/DHFR.

Figure 14 The upper part represents an outline of the mapping of RNA transcripts (↣) with nuclease S1. The lenght of the expected band is indicated (350 bp). The lower part is the x-ray exposure of the nuclease S1 mapping of RNA transcripts from cells infected with RVV-6 (pHGS-2/DHFR), RVV-7 (pHGS-A/DHFR) and RVV-8 (pHGS-F/DHFR). Early RNA was extracted 6 hours after infection of cells with the different viruses incubated in the presence of 100 µg/ml of cycloheximide (lanes indicated with +). Late RNA was extracted 8 and 24 hours post infection (lanes indicated with 8 resp. 24). The lane marked "M" consists of ^{32}P-labelled Hpa II fragments of pBR-322 giving the length position (in bp) as indicated. The positions of the S1 protected bands corresponding to RNA transcripts starting at the translocated (mutated) 11kDa regulatory sequences are indicated.

General methods

The following methods were performed as described by Maniatis et al., supra, unless indicated differently: Restriction endonuclease digestions at 37°C (pp. 100-101); dephosphorylation with bacterial alkaline phosphates (BAP) at 37°C (pp. 133-134): ligation with T4 DNA ligase at 14°C (pp. 390-391): transformation of DNA into $CaCl_2$-cells of E.coli HB101 and selection of tranformants on agar plates containing LB-medium plus 100 µg/ml of ampicillin (pp. 250-251); DNA plasmid preparation (pp. 86-94); filling-in single-stranded DNA-tails with the large fragment of DNA polymerase I (Klenow fragment) at 14°C (pp. 113-114); DNA separation and fragment purification from agarose gels (pp.164-167); the use of synthetic DNA linkers in subcloning (pp. 392-397); removal of single-stranded DNA-tails with nuclease S1 at room temperature (p. 140); isolation of mRNA from mammalian cells (pp. 191-193); nuclease-S1 mapping of mRNA (pp. 207-209); sequencing of DNA by the Maxam-Gilbert technique (pp. 475-478).
Cultured cells: Rabbit kidney (RK-13) (Christofinis. G. J. and Beale, A.J., J. Path.Bact. 95, 377-381 [1968]); human osteosarcoma cells transformed with murine sarcoma virus (Human TK$^-$143 cells repository no. GM 5887, Human genetic mutant Cell Repository, Institute for Medical Research, Copewood St., Camden, N.J.08103, USA). Maintenance of cells was at the indicated temperature in Eagle's minimal essential medium (E-MEM) supplemented with 5% fetal calf serum and 100 µg/ml of streptomycin and 100 IU/ml of penicillin at 80% humidity and 5% CO_2.

Example 1

Construction of recombinant vaccinia virus RVV-1 carrying the 11 kDa transcriptional regulatory sequence

A. Construction of plasmids pBR-J and pBR-F1 (Fig. 1a and 1b).

Ten µg of Vaccinia virus (VV) DNA (WR strain) were digested to completion with 100 units of the restriction endonuclease Hind III. The Hind III J-fragment (approx. 5 kb) containing the VV thymidine kinase (TK) gene (Weir, J.P. and Moss, B., J.Virology 46, 530-537 [1983]) and F-fragment (approx. 14 kb) were isolated from agarose. One µg of the plasmid pBR-322 (J.G. Sutcliffe, "Complete nucleotide sequence of the Escherichia coli plasmid pBR-322", Cold Spring Harbor Symp. Quant.Biol., 43, pp 77-90 [1979]) was digested with one unit of the restriction endonuclease Hind III to completion, free ends were dephosphorylated with one unit of bacterial alkaline phosphatase (BAP) for 1 h. Twenty ng of Hind III linearized pBR-322 and 100 ng of the Hind III J-fragment respectively F-fragment were ligated with one unit of T4 DNA ligase, transformed into E.coli HB101 cells. In each case four transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml ampicillin. DNA from these cultures was isolated using standard procedures and analyzed for its size and presence of sites for restriction endonucleases Hind III, EcoRI and Kpn I in the case of the J-fragment and Hind III, Sal I and Bgl I in the case of the F-fragment. One plasmid displaying the expected patterns after electrophoresis on agarose gels was designated pBR-J resp. pBR-F.

One µg of the plasmid pBR-F was digested to completion with 2 units of the restriction endonuclease Cla I, the DNA was loaded on a 0.8% agarose gel and a fragment of 5.6 kb (consisting of the plasmid pBR 322 and 1.25 kb of the insert) was isolated as described. Fifty ng of this plasmid were religated using 1 unit of T4 ligase and the DNA was transformed into HB101 as described. Four transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml ampicillin. DNA from these cultures was isolated using standard procedures and analyzed for its size and presence to sites for restriction endonucleases Hind III, Cla I and EcoRI. One plasmid displaying the expected patterns after electrophoresis on agarose gels was designated pBR-F1.

B. Construction of plasmid pUC-TK (Fig.2)

Two µg of the plasmid pBR-J were digested to completion with 5 units of the restriction endonuclease Hpa II and single-stranded DNA-tails were filled in with 4 units of the large fragment of DNA polymerase I (Klenow fragment). The DNA was subsequently digested to completion with 5 units of the restriction endocuclease Hind III, DNA separated on 0.8% agarose gel and a DNA fragment of 1310 bp was isolated from the agarose gel. Two µg of the plasmid pUC-8 (J.Vierra and J.Messing. "The pUC plasmids, an M13mp7- derived system for insertion mutagenesis and sequencing with synthetic-universal primers", Gene 19, pp 259-268 [1982]) were digested to completion with 5 units of the restriction endonuclease EcoRI and single-stranded DNA tails were removed with 2 units of nuclease S1 for 1 h at RT in S1 buffer. This DNA was subsequently digested to completion with 5 units of the restriction endonuclease Hind III, free ends dephosphorylated with 2 units of BAP for 1 h, the DNA was separated on a 0.8% agarose gel and a fragment of 2.7 kb was isolated from agarose as previously described. Fifty ng of this pUC-8 fragment were ligated with 200 ng of the purified 1310 bp VV fragment using one unit of T4 DNA ligase and the ligated DNA transformed into HP101. Eight transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml ampicillin. DNA from these cultures was isolated using standard procedures and analyzed for its size and presence of sites for restriction endonucleases Hind III, EcoRI and Xba I. One plasmid displaying the expected patterns after electrophoresis on agarose gels was designated pUC-TK.

C. Construction of plasmid pUC-TK/11kDa (Fig.3)

Fifty µg of the plasmid pBR-F1 were digested to completion with 200 units each of the restriction endonucleases EcoRI and Xba I, the DNA was separated on a 1.2% agarose gel. A DNA fragment of 104 bp was isolated, single-stranded DNA-tails were filled in with 4 units of Klenow fragment and synthetic EcoRI linkers (CGAATTCG) were attached. This new DNA fragment with Eco linkers (110 bp) consist of the 11kDa transcriptional regulatory sequence of VV. Two µg of the plasmid pUC-TK were digested to completion with 5 units of the restriction endocuclease EcoRI, free DNA-ends were dephosphorylated with 2 units of BAP, the DNA was separated on a 0.8% agarose gel and the linear DNA fragment of 4 kb was isolated and extracted. Fifty ng of the EcoRI linearized pUC-TK vector were ligated using 1 unit of T4 DNA ligase with 10 ng of the 110 bp promoter fragment and transformed into HB101. Eight transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of sites for restriction endonucleases EcoRI and Hph I. One plasmid displaying the expected patterns after electrophoresis on 6 % acrylamide gels was designated pUC-TK/11kDa.

D. Construction of plasmid pHGS-1 (Fig.4)

Ten µg of pUC-TK/11kDa were digested partially (up to 5%) with one unit of the restriction endonuclease EcoRI at 37°C for 30 min, single-stranded DNA-tails were filled in with 4 units of Klenow fragment at 14°C. The DNA was separated on a 0.8% agarose gel and the linearized form of the pUC-TK/11kDa plasmid (4104 bp) was isolated and 10 ng were self-ligated using 2 units of T4 DNA ligase and transformed into HB101. Sixteen transformants resistant to ampicillin were selected and cultures were grown in LB-medium containing 100 µg/ml of ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of sites for restriction endonucleases EcoRI, Xmn I and Hind III. Plasmids displaying the expected patterns after electrophoresis on agarose gel were selected and the Cla I-EcoRI fragment of 148 bp was sequenced. One plasmid displaying the sequence as indicated on page 3 preceeding and including the site for the restriction endonuclease EcoRI was designated pHGS-1.

E. Construction of recombinant vaccinia virus (RVV-1)

RK-13 cells adapted to 33 °C were infected with 0.1 plaque forming units (pfu) per cell of the vaccinia virus temperature sensitive mutant t_S 7 (Drillien, R. and Spehner, D., Virology 131, 385-393 [1983]). After 2 h at the permissive temperature of 33 °C, the cells were transfected with a calcium phosphate DNA precipitate as described (Weir, J.P. et al., Proc. Natl. Acad, Sci. USA. 79, 1210-1214 [1982]). Sixty ng of the vaccinia wild type DNA (WR strain) co-precipitated with 20 ng of the appropriate recombinant plasmids (pHGS-1) containing the transcriptional regulatory sequence of the 11kDa gene of vaccinia virus inserted into the body of the TK gene, were used per 2×10^5 cells. After two days of incubation at 39.5 °C the cells were disrupted by sonication, and the amount of TK negative (TK^-) virus in the progeny was determined by titration on Human-TK^-143-cells in the presence of 100 µg/ml bromodeoxyuridine. About a 200-fold increase in virus with a TK^- phenotype was found in cells that had been transfected with wild type DNA and the recombinant plasmids over that found in cells that had been transfected with wild type DNA alone. Plaques were picked and the virus was plaque-purified a second time on Human-TK^-143-cells in the presence of 30 µg/ml bromodeoxyuridine. Virus stocks were then made on RK-13 cells in the absence of bromodeoxyuridine. The virus RVV-1 was selected for the presence of the 11kDa promoter inserted into the TK-gene by blothybridization (Mackett. M., et al., Proc.Natl.Acad.Sci.USA. 79, 7415-7419 [1982]).

F. Cloning and preparation of the nuclease S1 probes

a. Nuclease S1 probe for transcripts of the 11kDa transcriptional regulatory sequence inserted in the TK-gene (Fig.5a)

Ten µg of the plasmids pHGS-1, were partially digested (up to 10%) with 2 units of the restriction endonuclease Taq I. The DNA's were subsequently digested to completion with 20 units of the restriction endonuclease Hind III separated on an agarose gel and a DNA fragment of 1135 bp was isolated. Hundred ng of this fragment were ligated with 1 unit of T4 DNA ligase with 25 ng of the plasmid pBR-322 digested to completion with 1 unit each of the restriction endonucleases Hind III and Cla I and transformed into HB101. Four transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml of ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of sites for restriction endonucleases Hind III, EcoRI and Cla I. Plasmids displaying the expected patterns on agarose gels were selected and used to prepare nuclease S1 DNA probes as followed: Ten µg of the plasmid were digested to completion with 20 units each of the restriction endonucleases Cla I and Hind III, free ends were dephosphorylated with 10 units of BAP, a DNA fragment of 1.6 kb was isolated and 0.1 pmol was labelled before use with 1 unit of polynucleotide kinase (PNK) and 1 pmol of γ-^{32}P-deoxyadenosine triphosphate, and the DNA was subsequently digested to completion with 1 unit of the restriction endonuclease RsA I.

b. Nuclease S1 probe for TK transcripts (Fig.5b)

Five µg of the plasmid pUC-TK were digested to completion with the restriction endonuclease ClaI, free ends were dephosphorylated with 5 units BAP, the DNA subsequently digested to completion with the restriction endonuclease Hind III, the DNA was separated on 0.8% low melting agarose and a DNA fragment of 737 bp was isolated. The S1 probe was labelled with PNK and γ-^{32}P-deoxyadenosine triphosphate as described.

G. 5' S1 mapping of the RNA transcripts

Two petridishes (5 cm Ø)with a confluent monolayer of RK-13 cells were infected each with 10 pfu per cell of the recombinant virus RVV-1 and after 3 respectively 7 h the RNA was extracted. The RNA's were resuspended in 100 µl of 0.2% SDS, divided into two equal parts and 20'000 cpm of the ^{32}P-labelled S1 probe for the TK-gene resp. 11KDa transcriptional regulatory sequence were added and the different mixtures were precipitated with 1/10 volume of NaOAc and 2 1/2 volumes of ethanol. The pellet was redissolved in 50 µl of S1 hybridisation buffer, heated for 3 minutes at 100 °C and incubated for 16 hours at 42 °C before addition of 450 µl of one times nuclease S1 buffer and 25 units of the enzyme nuclease S1. The digestion was stopped after one hour with EDTA (final concentration at 50 mM) and SDS (final concentration of 0.5%). Sodium hydroxide was added to a final concentration of 0.25 M and after 1 hour at 45 °C the mixture was neutralized to pH 7 using 3M HOAc. The residual DNA was precipitated using 5 µg of tRNA as a carrier, washed with 80% ethanol, dissolved in 20 µl formamide loading buffer and 10 µl were

loaded on a 8% acrylamide-8M urea sequencing gel. The gel was exposed for 24 hours using Kodak x-ray film and an intensifying screen. The x-ray exposure is shown in Fig.6. At 3 hours post infection, only transcripts from the TK-promoter can be detected (slot 1), whereas at 7 hours after infection a strong S1 protected band can be detected starting at the inserted 11kDa promoter (260 bp) and only trace amounts of the TK-transcripts. Therefore, it can be concluded that the inserted 11kDa regulatory sequence is functioning late after infection.

Example 2

Construction of recombinant vaccinia virus RVV-2 and RVV-3 carrying submits of the 11 kDa transcriptional regulatory sequence of VV.

A. Constructions of plasmids pHGS-2 and pHGD-2Δ15

Three µg of the plasmid pHGS-1 were digested to completion with 10 units of the restriction endonuclease EcoRI, the DNA was incubated at 37°C with 1 unit of T4 DNA polymerase in T4 polymerase buffer (Maniatis et al., supra) with deoxythymidine triphosphate at a final concentration of 100 µM. After 30 min the reaction was stopped by heating at 65°C for 10 min. The mixture was diluted 10-fold with nuclease S1 buffer and single-stranded DNA-tails were removed with 2 units of nuclease S1 for 1 hour. Synthetic Bam HI linkers (5'-AAAGGATCCTTT-3') were ligated to the blunt DNA-ends and 100 ng of the DNA was self-ligated with 1 unit of T4 DNA ligase and digested with 10 units of the restriction endonuclease EcoRI for 1 hour and transformed into HB101. Eighty transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml of ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of a site for restriction endonuclease Bam HI. DNA's containing a site for a restriction endonuclease Bam HI were selected. From each of these DNA's 20 µg were digested to completion with 50 units each of the restriction endonucleases Bam HI and Cla I. The DNA's were separated on acrylamide gels and DNA fragments of 156 resp. 141 bp were isolated and sequenced using the Maxam-Gilbert technique after labelling the fragments at the site of the restriction endonuclease Bam I using 1 unit of Klenow fragment and α-^{32}P-deoxyguanidine triphosphate. DNA's were selected having the following sequence preceeding the sequence 5'-AAAGGATCC-3' (representing the Bam HI linker) :

```
No 1: 5'-  CTAGA AGCGA TGCTA CGCTA GTCAC AATCA CCACT TTCAT
           ATTTA GAATA TATGT ATGTA AAAAT ATAGT AGAAT TTCAT
           TTTGT TTTTT TCTAT GCTAT AAAT -3'

No 2: 5'-  CTAGA AGCGA TGCTA CGCTA GTCAC AATCA CCACT TTCAT
           ATTTA GAATA TATGT ATGTA AAAAT ATAGT AGAAT TTCAT
           TTTGT TTTTT -3'
```

Five µg of the DNA's having the sequence No 1 resp. 2 were digested to completion with 10 units each of the restriction endonucleases Bam HI and Hind III. The DNA was separated on an agarose gel and DNA fragments of 888 resp. 873 were isolated, and 100 ng each were ligated with a 50-molar excess each of the synthetic DNA fragments 5'-GATCCCCGGG-3' and 5'-AATTCCCGGG-3' using 1 unit of T4 DNA ligase. The DNA was subsequently digested with 300 units of the restriction endonuclease EcoRI for 8 h, loaded on a 6% acrylamide gel and fragments of 898 resp. 883 were isolated.

Two µg of the plasmid pHGS-1 were digested to completion with 5 units each of the restriction endonucleases EcoRI and Hind III, the DNA was separated on an agarose gel and a fragment of 3.2 kb was isolated. Twenty five ng of this 3.2 kb fragment were ligated with 50 ng of the 898 resp. 883 DNA fragments using 1 unit of T4 DNA ligase and the DNA's transformed into HB101. In each case 4 transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml of ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of sites for restriction endonucleases EcoRI, Sma I and Bam HI. In each case one plasmid displaying the expected patterns after electrophoresis on agarose gels was designated pHGS-2 (containing sequence No 1) resp. pHGS-2Δ15 (No

2).

B. Construction of recombinant virus RVV-2 and RVV-3

The recombinant viruses RVV-2 resp. RVV-3 were constructed as described before using the plasmids pHGS-2 resp. pHGS-2Δ15. In each case one virus was selected for the presence of the mutated 11kDa transcriptional regulatory sequence (No 1 resp. 2) inserted into the TK-gene by blot-hybridization (Mackett et al., supra).

C. Cloning of the nuclease S1 probes

The preparation of the nuclease S1 probes for transcripts of the transcriptional regulatory sequence No 1 resp. No 2 inserted into the TK-gene was as described in example 1 section F.a using the plasmids pHGS-2 resp. pHGS-2Δ15.

D. 5' S1 mapping of the RNA transcripts

RNA transcripts 4 resp. 8 h after virus infection were prepared as described previously using the viruses RVV-2 resp. RVV-3 and the S1 probes for RNA transcripts from the TK promoter resp. 11kDa transcriptional regulatory sequences (containing sequences No 1 resp. 2). The x-ray exposure is shown in Fig.7. At 4 hours post infection (slot 1 and 4) only transcripts from the TK promoter can be detected. These transcripts are undetectable at 8 hours post infection, whereas cells infected with the virus RVV-2 (pHGS-2 inserted) a strong S1 protected band can be detected. However with RVV-3 (pHGS-2Δ15 inserted) no S1 protected band appears, demonstrating that the deletion of 15 basepairs 5' from the ATG results in an inactivation of transcripts starting from the mutated transcriptional regulatory sequence.

Example 3

Construction of recombinant vaccinia virus RVV-4 and RVV-5 containing the merozoite 5.1 surface antigen operatively linked to the 11 kDa transcriptional regulatory sequence of VV.

A. Cloning of Plasmodium falciparum 5.1 Antigen (Fig 8)

Hundred ng of a Plasmodium falciparum cDNA fragment with flanking EcoRI-linkers, containing the merozoite 5.1 surface antigen of Plasmodium falciparum (Hope, I. A. et al., Nucleic Acids Research, 13, 369-379 [1985]) were ligated using 1 unit of T4 DNA ligase into 50 ng of pHGS-2 resp. pHGS-2Δ15, digested to completion with the restriction endonuclease EcoRI, free ends were dephosphorylated using 1 unit of BAP. The ligated DNA's were subsequently transformed into HB101 and in each case four transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of sites for restriction endonucleases EcoRI and Hinc II. In each case one plasmid displaying the expected patterns after electrophoresis on agarose gels was designated pHGS-2/5.1 resp. pHGS-2Δ15/5.1.

B. Construction of recombinant vaccinia virus RVV-4 and RVV-5

The recombinant viruses RVV-4 and RVV-5 were constructed and selected as described before using the plasmids pHGS-2/5.1 and pHGS-2Δ15/5.1

C. Indirect immunofluorescence

RK-13 cells were grown upto 80% confluency and infected with 0.1 plaque forming units (pfu) per cell of recombinant vaccinia virus RVV-4 resp. RVV-5 with the malaria 5.1 Antigen stabely integrated in the virus genome through homologous recombination. One hour after infection the cells were washed with phosphate buffered saline (PBS) and fresh medium was added to the cells. Infection with the virus was allowed to continue for additional 16 hours. Cells were scraped of the culture dishes, harvested through centrifugation at 2000xg and washed once with PBS. Approximately 10^4 cells were spotted on a microscope glass, air dried, fixed with -20 °C acetone for 10 min and again air dried. The fixed cells were incubated at 37 °C for 20 min with rabbit anti-5.1 antigen diluted in PBS, subsequently washed 3 times with PBS and again air

dried. Next the fixed cells were incubated at 37°C for 20 min with goat anti-rabbit FITC serum diluted with PBS, washed 3 times with PBS, once with distilled water and air dried. PBS-glycerin (1:1) was spotted on the cells and covered with a microscope cover-glass. The cells were analyzed for their fluorescence under a microscope at 450-490 nm. The result is shown in Fig.9. Panel A shows fluorescent cells infected with the virus RVV-4 and panel B infected cells in visible light. Cells infected with the virus RVV-5 did not show any fluorescence, demonstrating that deletions upstream of the 11kDa-ATG causes inactivation of the regulatory sequences. Removal of the G residue of the ATG however has no effect on transcription or translation.

EXAMPLE 4

Construction of recombinant vaccinia virus RVV-6, RVV-7 and RVV-8 containing the mouse dihydrofolate reductase operatively linked to mutated 11kDa transcriptional regulatory sequences of VV.

A. Construction of plasmid pHGS-2/DHFR (Fig. 10)

Two µg of the plasmid pHGS-2 were digested to completion with 2 units each of the restriction endonucleases Bam HI and EcoRI. The DNA was separated on an 0.8% agarose gel and the Bam HI-EcoRI vector was isolated (~ 4 kb).

Two µg of the plasmid pDS-1, to 1^+ (deposited at Deutsche Sammlung von Mikroorganismen (DSM) in Göttingen on December 11, 1984, accession no. DSM 3135) were digested to completion with two units each of restriction endonucleases Bam HI and EcoRI and a fragment of approximately 920 bp containing the mouse dihydrofolate reductase (DHFR) gene was isolated. Twenty µg of the Bam HI and EcoRI digested vector pHGS-2 and 100 µg of the Bam HI-EcoRI fragment containing the mouse dihydrofolate reductase gene were ligated using 1 unit of T4 DNA ligase and the DNA was transformed into HB 101. Eight transformants resistant to 100 µg/ml of ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml of ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of sites for restriction endonucleases EcoRI, Bam H and SacI. One plasmid displaying the expected patterns was designated pHGS-2/DHFR.

B. Construction of plasmid pHGS-2/DHFR-E (Fig. 11)

Two µg of the plasmid pHGS-2 were digested to completion with two units of the restriction endonuclease EcoRI, the free ends were dephosphorylated with one unit of Bacterial Alkaline Phosphatase (BAP) and the EcoRI digested vector was isolated from low-melting (LM) agarose. Two µg of the plasmid pDS-1, to 1^+ were digested to completion with two units of the restriction endonuclease EcoRI and a fragment of approximately 920 bp containing the mouse DHFR-gene was isolated from LM agarose. Twenty µg of the EcoRI digested vector pHGS-2 and 100 µg of the EcoRI-fragment were ligated with one unit of T4 ligase and the DNA was transformed into HB 101. Eight transformants resistent to 100 µg/ml of ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml of ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of sites for restriction endonucleases EcoRI and SacI. One plasmid displaying the expected patterns after electrophoresis on agarose gels was designated pHGS-2/DHFR-E (Fig. 11).

C. Construction of plasmid pHGS-A/DHFR (Fig. 12)

Ten µg of the plasmid pHGS-2/DHFR-E were digested to completion with ten units each of the restriction endonucleases EcoRI and SacI and a fragment of approximately 275 bp was isolated from 8% acrylamide gel. Two hundred µg of the 275 bp EcoRI-SacI fragment were ligated with 50 pmol each of the synthetic fragment 5'-TATAAATA-3' and 5'-AATTTATTTATA-3' in ligase buffer containing 50 mM NaCl using one unit of T4 DNA ligase. After ligation for 2 hours at 14°C, the DNA was digested to completion with one unit of restriction endonuclease SacI and a fragment of 283 bp containing the above mentioned synthetic sequences was isolated.

Two µg of the plasmid pHGS-2Δ15 were digested to completion with four units of restriction endonuclease Bam H and the 5' overhangs were filled in with a large fragment of the E. coli DNA polymerase I (Klenow fragment) and the enzyme was inactivated through incubation at 65°C, for 10 minutes. Subsequently the DNA was digested to completion with one unit of ClaI and a fragment of 152 bp was isolated from a 8% acrylamide gel.

Two µg of the plasmid pHGS-2/DHFR were digested to completion with 2 units each of the restriction

endonuclease ClaI and SacI and the vector of approximately 4.6 kb was isolated from LM agarose. Hundred µg of the EcoRI-SacI fragment containing the indicated synthetic fragments and hundred µg of the ClaI-Bam HI fragment containing part of the 11 KDa transcriptional regulatory sequence were ligated with 20 µg of the ClaI-SacI digested vector pHGS-2/DHFR using one unit of T4 DNA ligase and the DNA was transformed into HB 101. 16 transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml of ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of sites for restriction endonucleases EcoRI and SacI. One plasmid displaying the expected patterns after electrophoresis on acrylamide gels was sequenced. The plasmid was designated pHGS-A/DHFR containing the sequence:
No. 3:

```
5'- CTAGA AGCGA TGCTA CGCTA GTCAC AATCA CCACT TTCAT
    ATTTA GAATA TATGT ATGTA AAAAT ATAGT AGAAT TTCAT
    TTTGT TTTTT AAAGG ATCTA TAAAT AAAT 3'
```

D. Construction of plasmid pHGS-F/DHFR (Fig. 13)

Ten µg of the plasmid pHGS-2/DHFR were digested to completion with 10 units each of the restriction endonucleases Bam HI and SacI and a fragment of 268 bp was isolated from a 8% acrylamide gel. Two hundred µg of the Bam HI-SacI fragment were ligated with 50 pmol each of the synthetic fragments 5'-TCTATCGATTAAATAAA-3' and 5'-GATCTTTATTTAATCGATAGA-3' in ligase buffer containing 50 mM NaCl final concentration using one unit of T4 DNA ligase. After ligation for 2 hours at 14 °C the DNA was digested with one unit of the restriction endonuclease SacI and the fragment of 290 bp containing the above mentioned sequence was isolated. Ten µg of the plasmid pHGS-2 Δ15 were digested to completion with 10 units each of the restriction endonucleases ClaI and DraI and a fragment of 130 bp was isolated from a 8% acrylamid gel.

Hundred µg each of the Bam HI-SacI fragment containing the indicated synthetic fragments and the ClaI-DraI fragment containing part of the 11KDa transcriptional regulatory sequence were ligated with 50 µg of the ClaI-SacI digested vector pHGS-2/DHFR using one unit of T4 DNA ligase and the DNA was transformed into HB 101. Sixteen transformants resistant to ampicillin were selected and cultures grown in LB-medium containing 100 µg/ml of ampicillin. DNA from these cultures was isolated and analyzed for its size and presence of sites for restriction endonucleases SacI and ClaI. One plasmid displaying the expected patterns after electrophoresis on acrylamide gels was sequenced. The plasmid was designated pHGS-F/DHFR containing the sequence:
No 4:

```
5'- CTAGA AGCGA TGCTA CGCTA GTCAC AATCA CCACT TTCAT
    ATTTA GAATA TATGT ATGTA AAAAT ATAGT AGAAT TTCAT
    TTTGT TTTTT TCTAT CGATT AAATA AAG  3'
```

E. Construction of recombinant virus RVV-6, RVV-7 and RVV-8

The recombinant viruses RVV-6, RVV-7 resp. RVV-8 were constructed as described before using the plasmids pHGS-2/DHFR, pHGS-A/DHFR and pHGS-F/DHFR. In each case one virus was selected for the presence of the chimeric gene consisting of the mutated 11 kDa transcriptional regulatory sequence (No.1, 3 resp. 4) and DHFR gene inserted into the VV TK-gene by blot-hybridization (Mackett et al., supra).

F. Preparation of nuclease S1 probes

10 µg of each of the plasmids pHGS-2/DHFR, pHGS-A/DHFR and pHGS-F/DHFR were digested to completion with 10 units of the restriction endonuclease AccI, the free-ends were dephosphorylated using one unit of BAP and the enzyme was removed through subsequent extractions with phenol, phenol-chloroform (1:1,v/v) and chloroform. The DNA's were precipitated and the free-ends were phosphorylated using polynucleotide kinase in the presence of 2-fold molar excess of ^{32}P-γ-ATP. The enzyme was inactivated at 65° C for 10 minutes and the DNA's were digested to completion with 5 units of the restriction endonuclease HindIII. Fragments of approximately 1200 bp were isolated from LM agarose representing the S1 probes asymmetrically labeled at the AccI restriction endonuclease site.

G. 5' S1 mapping of the RNA transcripts

Monolayers of RK-13 cells were infected with 5 pfu per cell of the recombinant viruses RVV-6, RVV-7 resp. RVV-8. Early RNA was prepared 6 hours after infection of the cells incubated in medium containing 100 µg/ml of cycloheximide (Sigma). Late RNS's were isolated from virus infected cells 8 and 24 hours post-infection. S1 mapping was performed as described in Example 1, section G, using the labelled S1 probes for the DHFR transcripts prepared as described in section F.

The x-ray exposure is shown in Fig. 14. DHFR transcripts are not detectable in RNA prepared from infected cells incubated in the presence of cycloheximide, indicating that the 11KDa regulatory sequences no. 1, 3 and 4 are not active early in transcription (Fig. 14, lanes indicated with +).

DHFR transcripts (indicated by arrow) are detectable 8 and 24 hours after infection in cells infected with the viruses RVV-6, RVV-7 resp. RVV-8. (Fig. 14, lanes indicated with 8 resp. 24). The amounts of DHFR transcripts derived from the mutated 11KDa regulatory sequences no. 3 and 4 present in the insertion vectors pHGS-A/DHFR resp. pHGS-F/DHFR are at least 3- to 4-fold increased compared to the DHFR transcripts derived from the 11KDa regulatory sequence no. 1 present in the insertion vector pHGS-2/DHFR.

Claims

1. A transcriptional regulatory sequence of formula

5' CTAGA AGCGA TGCTA

CGCTA GTCAC AATCA CCACT

TTCAT ATTTA GAATA TATGT

ATGTA AAAAT ATAGT AGAAT

TTCAT TTTGT TTTTT TCTAT

GCTAT AAATG 3'

or subunits thereof which contain the genetic information to function as a poxvirus late promoter.

2. A transcriptional regulatory sequence as claimed in claim 1 of formula

```
5'        CTAGA     AGCGA     TGCTA

     CGCTA     GTCAC     AATCA     CCACT

     TTCAT     ATTTA     GAATA     TATGT

     ATGTA     AAAAT     ATAGT     AGAAT

     TTCAT     TTTGT     TTTTT     TCTAT

     GCTAT     AAAT      3'
```

3. A transcriptional regulatory sequence as claimed in claim 1 of formula

```
5'        CTAGA     AGCGA     TGCTA

     CGCTA     GTCAC     AATCA     CCACT

     TTCAT     ATTTA     GAATA     TATGT

     ATGTA     AAAAT     ATAGT     AGAAT

     TTCAT     TTTGT     TTTTT     AAAGG

     ATCTA     TAAAT     AAAT      3'
```

4. A transcriptional regulatory sequence as claimed in claim 1 of formula

5' CTAGA AGCGA TGCTA

CGCTA GTCAC AATCA CCACT

TTCAT ATTTA GAATA TATGT

ATGTA AAAAT ATAGT AGAAT

TTCAT TTTGT TTTTT TCTAT

CGATT AAATA AAG 3'

5. A transcriptional regulatory sequence as claimed in claim 1 of formula

5' CTAT GCTAT AAAT 3'

6. A recombination vector comprising
(a) the vector origin of replication
(b) an antibiotic resistance gene
(c) a chimeric gene consisting of at least one transcriptional regulatory sequence as claimed in any one of claims 1-5 operatively linked to a foreign gene encoding prokaryotic or eukaryotic polypeptides and
(d) DNA from a non-essential segment of the poxvirus genome flanking said chimeric gene.

7. A recombination vector according to claim 6 wherein the translational initiation site of the chimeric gene is provided by the poxvirus transcriptional regulatory sequence.

8. A recombination vector according to claim 6 wherein the translational initiation site of the chimeric gene is provided by the foreign gene encoding a prokaryotic or eukaryotic polypeptide.

9. A recombination vector according to any one of claims 6-8 which is a plasmid capable of replication in gram-negative bacteria.

10. A recombination vector according to claim 9 which is capable of replication in an E. coli strain.

11. A recombination vector according to any one of claims 6 and 8-10, wherein said foreign gene encodes a malaria antigen.

12. A recombination vector according to claim 11 wherein the malaria antigen is a sporozoite and/or merozoite surface antigen of Plasmodium falciparum.

13. A recombination vector according to claim 12 wherein the malaria antigen is the 5.1 antigen.

14. A recombination vector according to any one of claims 6-13 wherein said poxvirus DNA is vaccinia virus DNA.

15. A recombination vector according to claim 14 which is pHGS-1 as contained in E. coli HB 101 having

the accession number DSM 3248.

16. A recombination vector according to claim 14 which is pHGS-2 as contained in E. coli HB 101 having the accession number DSM 3249.

17. A recombinant infectious poxvirus containing a chimeric gene consisting of a transcriptional regulatory sequence as claimed in any one of claims 1-5 operatively linked to a foreign gene encoding prokaryotic or eukaryotic polypeptides and capable of expressing said foreign gene.

18. A recombinant infectious poxvirus according to claim 17 wherein the translational inition site of the chimeric gene is provided by the poxvirus transcriptional regulatory sequence.

19. A recombinant infectious poxvirus according to claim 17 wherein the translational inition site of the chimeric gene is provided by the foreign gene encoding prokaryotic or eukaryotic polypeptides.

20. A recombinant infectious poxvirus according to claim 17 and 19 wherein said foreign gene encodes a malaria antigen.

21. A recombinant infectious poxvirus according to claim 20 wherein the malaria antigen is a sporozoite and/or merozoite surface antigen of Plasmodium falciparum.

22. A recombinant infectious poxvirus according to claim 21 wherein the malaria antigen is the 5.1 antigen.

23. A recombinant infectious poxvirus according to any one of claims 17-22 which is an infectious recombinant vaccinia virus.

24. An infectious vaccinia virus according to claim 23 which is RVV-1 obtainable by transfection of an eukaryotic host cell with recombination vector pHGS-1 as defined in claim 15.

25. An infectious vaccinia virus according to claim 23 which is RVV-2 obtainable by transfection of an eukaryotic host cell with recombination vector pHGS-2 as defined in claim 16.

26. A method for the manufacture of a recombination vector as claimed in any one of claims 6-16 comprising the steps of:
(a) preparing a vector containing poxvirus DNA, said DNA comprising:
(i) at least one transcriptional regulatory sequence next to at least one restriction endonuclease site, and
(ii) DNA from a non-essential segment of the poxvirus genome flanking said regulatory sequence and said restriction endonuclease site; and
(b) inserting at least one foreign gene encoding a prokaryotic or eukaryotic polypeptide into said restriction endonuclease site next to said transcriptional regulatory sequence.

27. The method according to claim 26 wherein said poxvirus DNA is vaccinia virus DNA.

28. A method for the manufacture of a recombinant infectious poxvirus as claimed in any one of claims 17-25 comprising the steps of:
(a) preparing a recombination vector according to claim 26 or claim 27;
(b) providing at least one cell infected with a virus from the genus of poxvirus;
(c) transfecting said cell with said recombination vector, whereby homologous recombination occurs between the DNA of said poxvirus and at least one portion of said poxvirus DNA contained in said recombination vector; and
(d) isolating from said cell a recombinant infectious poxvirus capable of expressing said foreign gene encoding prokaryotic or eukaryotic polypeptides by selective methods.

29. The method according to claim 28 wherein said poxvirus DNA is vaccinia virus DNA.

30. Live vaccines containing a recombinant infectious poxvirus as claimed in any one of claims 17-23 and a physiologically acceptable carrier.

31. The use of a recombinant infectious poxvirus as claimed in any one of claims 17-25 for protective immunization.

Revendications

1. Séquence régulatrice de transcription de formule

```
5'        CTAGA     AGCGA     TGCTA

   CGCTA  GTCAC     AATCA     CCACT

   TTCAT  ATTTA     GAATA     TATGT

   ATGTA  AAAAT     ATAGT     AGAAT

   TTCAT  TTTGT     TTTTT     TCTAT

   GCTAT  AAATG     3'
```

ou ses sous-unités qui contiennent l'information génétique afin de fonctionner comme promoteur tardif de poxvirus.

2. Séquence régulatrice de transcription selon la revendication 1, de formule

```
5'        CTAGA     AGCGA     TGCTA

   CGCTA  GTCAC     AATCA     CCACT

   TTCAT  ATTTA     GAATA     TATGT

   ATGTA  AAAAT     ATAGT     AGAAT

   TTCAT  TTTGT     TTTTT     TCTAT

   GCTAT  AAAT      3'
```

3. Séquence régulatrice de transcription selon la revendication 1 de formule

5' CTAGA AGCGA TGCTA

CGCTA GTCAC AATCA CCACT

TTCAT ATTTA GAATA TATGT

ATGTA AAAAT ATAGT AGAAT

TTCAT TTTGT TTTTT AAAGG

ATCTA TAAAT AAAT 3'

4. Séquence régulatrice de transcription selon la revendication 1 de formule

5' CTAGA AGCGA TGCTA

CGCTA GTCAC AATCA CCACT

TTCAT ATTTA GAATA TATGT

ATGTA AAAAT ATAGT AGAAT

TTCAT TTTGT TTTTT TCTAT

CGATT AAATA AAG 3'

5. Séquence régulatrice de transcription selon la revendication 1 de formule

5' CTAT GCTAT AAAT 3'

6. Vecteur de recombinaison comprenant
   (a) le vecteur origine de réplication
   (b) un gène de résistance aux antibiotiques
   (c) un gène chimère constitué d'au moins une séquence régulatrice de transcription selon l'une quelconque des revendications 1-5 liée de manière opératoire à un gène étranger codant pour des polypeptides procaryotiques ou eucaryotiques et
   (d) de l'ADN provenant d'un segment non-essentiel du génome de poxvirus avoisinant ledit gène chimère.

7. Vecteur de recombinaison selon la revendication 6 dans lequel le site d'initiation de traduction du gène chimère est fourni par la séquence régulatrice de transcription du poxvirus.

8. Vecteur de recombinaison selon la revendication 6 dans lequel le site d'initiation de traduction du gène chimère est fourni par le gène étranger codant pour un polypeptide procaryotique ou eucaryotique.

9. Vecteur de recombinaison selon l'une quelconque des revendications 6-8 qui est un plasmide capable de réplication dans des bactéries gram-négatives.

10. Vecteur de recombinaison selon la revendication 9 qui est capable de réplication dans une souche d'E.coli.

11. Vecteur de recombinaison selon l'une quelconque des revendications 6 et 8-10, dans lequel ledit gène étranger code pour un antigène de la malaria.

12. Vecteur de recombinaison selon la revendication 11 dans lequel l'antigène de la malaria est un antigène de surface sporozoïte et/ou mérozoïte de Plasmodium falciparum.

13. Vecteur de recombinaison selon la revendication 12 dans lequel l'antigène de la malaria est l'antigène 5.1.

14. Vecteur de recombinaison selon l'une quelconque des revendications 6-13 dans lequel ledit ADN de poxvirus est l'ADN du virus de la vaccine.

15. Vecteur de recombinaison selon la revendication 14 qui est pHGS-1 tel que contenu dans E. coli HB 101 ayant le N° DSM 3248.

16. Vecteur de recombinaison selon la revendication 14 qui est pHGS-2 tel que contenu dans E. coli HB 101 ayant le N° DSM 3249.

17. Poxvirus infectieux recombinant contenant un gène chimère constitué d'une séquence régulatrice de transcription selon l'une quelconque des revendications 1-5 liée de façon opératoire à un gène étranger codant pour des polypeptides procaryotiques ou eucaryotiques et capable d'exprimer ledit gène étranger.

18. Poxvirus infectieux recombinant selon la revendication 17 dans lequel le site d'initiation de traduction du gène chimère est fourni par la séquence régulatrice de transcription du poxvirus.

19. Poxvirus infectieux recombinant selon la revendication 17 dans lequel le site d'initiation de traduction du gène chimère est fourni par le gène étranger codant pour les polypeptides procaryotiques ou eucaryotiques.

20. Poxvirus infectieux recombinant selon les revendications 17 et 19 dans lequel ledit gène étranger code pour un antigène de la malaria.

21. Poxvirus infectieux recombinant selon la revendication 20 dans lequel l'antigène de la malaria est un antigène de surface sporozoïte et/ou mérozoïte de Plasmodium falciparum.

22. Poxvirus infectieux recombinant selon la revendication 21 dans lequel l'antigène de la malaria est l'antigène 5.1.

23. Poxvirus infectieux recombinant selon l'une quelconque des revendications 17-22 qui est un virus de la vaccine recombinant infectieux.

24. Virus de la vaccine infectieux selon la revendication 23 qui est RVV-1 que l'on peut obtenir par transfection d'une cellule hôte eucaryotique avec le vecteur de recombinaison pHGS-1 tel que défini dans la revendication 15.

25. Virus de la vaccine infectieux selon la revendication 23 qui est RVV-2 que l'on peut obtenir par transfection d'une cellule hôte eucaryotique avec le vecteur de recombinaison pHGS-2 tel que défini dans la revendication 16.

26. Procédé de préparation d'un vecteur de recombinaison selon l'une quelconque des revendications 6-16 comprenant les étapes de:
(a) préparation d'un vecteur contenant l'ADN du poxvirus, ledit ADN comprenant:
(i) au moins une séquence régulatrice de transcription au voisinage d'au moins un site d'endonucléase de restriction, et
(ii) l'ADN d'un segment non-essentiel du génome du poxvirus avoisinant ladite séquence régulatrice et ledit site d'endonucléase de restriction; et
(b) insertion d'au moins un gène étranger codant pour un polypeptide procaryotique ou eucaryotique dans ledit site d'endonucléase de restriction au voisinage de ladite séquence régulatrice de transcription.

27. Procédé selon la revendication 26 dans lequel ledit ADN de poxvirus est l'ADN du virus de la vaccine.

28. Procédé de préparation d'un poxvirus infectieux recombinant selon l'une quelconque des revendications 17-25 comprenant les étapes de:
(a) préparation d'un vecteur de recombinaison selon la revendication 26 ou la revendication 27;
(b) fourniture d'au moins une cellule infectée avec un virus du genre des poxvirus;
(c) transfection de ladite cellule avec ledit vecteur de recombinaison, par lequel une recombinaison homologue s'effectue entre l'ADN dudit poxvirus et au moins une partie dudit ADN de poxvirus contenu dans ledit vecteur de recombinaison; et
(d) isolement dans ladite cellule d'un poxvirus infectieux recombinant capable d'exprimer ledit gène étranger codant pour des polypeptides procaryotiques ou eucaryotiques par des procédés sélectifs.

29. Procédé selon la revendication 28 dans lequel ledit ADN de poxvirus est l'ADN du virus de la vaccine.

30. Vaccins vivants contenant un poxvirus infectieux recombinant selon l'une quelconque des revendications 17-23 et un support physiologiquement acceptable.

31. Application d'un poxvirus infectieux recombinant selon l'une quelconque des revendications 17-25 à une immunisation protectrice.

Patentansprüche

1. Eine Transkriptionsregulations-Sequenz mit der Formel

5' CTAGA AGCGA TGCTA

CGCTA GTCAC AATCA CCACT

TTCAT ATTTA GAATA TATGT

ATGTA AAAAT ATAGT AGAAT

TTCAT TTTGT TTTTT TCTAT

GCTAT AAATG 3'

oder Untereinheiten davon, welche die genetische Information enthalten um als ein später Pockenvirus Promotor zu wirken.

2. Eine Transkriptionsregulations-Sequenz gemäss Anspruch 1 mit der Formel

5' CTAGA AGCGA TGCTA

CGCTA GTCAC AATCA CCACT

TTCAT ATTTA GAATA TATGT

ATGTA AAAAT ATAGT AGAAT

TTCAT TTTGT TTTTT TCTAT

GCTAT AAAT 3'

3. Eine Transkriptionsregulations-Sequenz gemäss Anspruch 1 mit der Formel

5' CTAGA AGCGA TGCTA

CGCTA GTCAC AATCA CCACT

TTCAT ATTTA GAATA TATGT

ATGTA AAAAT ATAGT AGAAT

TTCAT TTTGT TTTTT AAAGG

ATCTA TAAAT AAAT 3'

4. Eine Transkriptionsregulations-Sequenz gemäss Anspruch 1 mit der Formel

5' CTAGA AGCGA TGCTA

CGCTA GTCAC AATCA CCACT

TTCAT ATTTA GAATA TATGT

ATGTA AAAAT ATAGT AGAAT

TTCAT TTTGT TTTTT TCTAT

CGATT AAATA AAG 3'

5. Eine Transkriptionsregulations-Sequenz gemäss Anspruch 1 mit der Formel

5' CTAT GCTAT AAAT 3'

6. Eine Rekombinationsvektor enthaltend
(a) den vektoriellen Replikationsstartpunkt,
(b) ein Antibiotikaresistenzgen,
(c) ein chimäres Gen, welches aus wenigstens einer Transkriptionsregulations-Sequenz gemäss den Ansprüchen 1 - 5 besteht, die operativ mit einem Fremdgen, welches pro - oder eukaryotische Polypeptide kodiert, verknüpft ist, und
(d) DNA von nicht-essentiellen Bereichen des Pockenvirus Genoms, welche besagtes chimäres Gen flankiert.

7. Ein Rekombinationsvektor gemäss Anspruch 6, worin der Translationsstartort des chimären Gens von der Pockenvirvs Transkriptionsregulations-Sequenz geliefert wird.

8. Ein Rekombinationsvektor gemäss Anspruch 6, worin der Translationsstartort des chimären Gens vom Fremdgen, welches pro- oder eukaryotische Polypeptide kodiert, geliefert wird.

9. Ein Rekombinationsvektor gemäss einem der Ansprüche 6 - 8, der ein Plasmid ist, welches in gram-negativen Bakterien replizieren kann.

10. Ein Rekombinationsvektor gemäss Anspruch 9, der in einem E. coli Stamm replizieren kann.

11. Ein Rekombinationsvektor gemäss einem der Ansprüche 6 und 8 - 10, worin besagtes Fremdgen ein Malaria Antigen kodiert.

12. Ein Rekombinationsvektor gemäss Anspruch 11, worin das Malaria Antigen ein sporozoiten und/oder merozoiten Oberflächenantigen von Plasmodium falciparum ist.

13. Ein Rekombinationsvektor gemäss Anspruch 12, worin das Malaria Antigen das 5.1 Antigen ist.

14. Ein Rekombinationsvektor gemäss einem der Ansprüche 6 - 13, worin besagte Pockenvirus DNA Vaccinia Virus DNA ist.

15. Ein Rekombinationsvektor gemäss Anspruch 14 mit der Bezeichnung pHGS-1, wie in E. coli HB 101

mit der Hinterlegungsnummer DSM 3248 enthalten.

16. Ein Rekombinationsvektor gemäss Anspruch 14 mit der Bezeichnung pHGS2, wie in E. coli HB 101 mit der Hinterlegungsnummer DSM 3249 enthalten.

17. Ein rekombinanter, infektiöser Pockenvirus, welcher ein chimäres Gen enthält, das aus einer Transkriptionsregulations-Sequenz gemäss einem der Ansprüche 1 - 5 steht, die operativ mit einem Fremdgen, welches pro - oder eukaryotische Polypeptide kodiert, verknüpft ist und welcher in der Lage ist besagtes Fremdgen zu exprimieren.

18. Ein rekombinanter, infektiöser Pockenvirus gemäss Anspruch 17, worin der Translationsstartort des chimären Gens von der Pockenvirus Transkriptionsregulations-Sequenz geliefert wird.

19. Ein rekombinanter, infektiöser Pockenvirus gemäss Anspruch 17, worin der Translationsstartort des chimären Gens vom Fremdgen, welches pro-oder eukaryotische Polypeptide kodiert, geliefert wird.

20. Ein rekombinanter, infektiöser Pockenvirus gemäss Anspruch 17 und 19, worin besagtes Fremdgen ein Malaria Antigen kodiert.

21. Ein rekombinanter, infektiöser Pockenvirus gemäss Anspruch 20, worin das Malaria Antigen ein sporozoiten und/oder merozoiten Oberflächenantigen von Plasmodium falciparum ist.

22. Ein rekombinanter, infektiöser, Pockenvirus gemäss Anspruch 21, worin das Malaria Antigen das 5.1 Antigen ist.

23. Ein rekombinanter, infektiöser Pockenvirus gemäss einem der Ansprüche 17 - 22, der ein infektiöser, rekombinanter Vaccinia Virus ist.

24. Ein infektiöser Vaccinia Virus gemäss Anspruch 23 mit der Bezeichnung RVV-1, erhältlich durch Transfektion einer eukaryotischen Wirtszelle mit dem wie in Anspruch 15 definierten Rekombinationsvektor pHGS-1.

25. Ein infektiöser Vaccinia Virus gemäss Anspruch 23 mit der Bezeichnung RVV-2, erhältlich durch Transfektion einer eukaryotischen Wirtszelle mit dem wie in Anspruch 16 definierten Rekombinationsvektor pHGS-2.

26. Ein Verfahren zur Herstellung eines Rekombinationsvektors gemäss einem der Ansprüche 6 - 16, welches die Schritte einschliesst:
    (a) Herstellung eines Vektors, der Pockenvirus DNA enthält, welche
        (i) wenigstens eine Transkriptionsregulations-Sequenz neben einer Restriktionsendonuklease Schnittstelle, und
        (ii) DNA von nicht-essentiellen Bereichen des Pockenvirus Genoms, welche besagte Regulationssequenz und besagte Restriktionsendonuklease Schnittstelle flankiert, einschliesst; und
    (b) Einbau wenigstens eines Fremdgens, welches pro-oder eukaryotische Polypeptide kodiert, in die besagte Restriktionsendonuklease Schnittstelle neben besagter Transkriptionsregulations-Sequenz.

27. Das Verfahren gemäss Anspruch 26, worin besagte Pockenvirus DNA Vaccinia Virus DNA ist.

28. Ein Verfahren zur Herstellung eines rekombinanten, infektiösen Pockenvirus gemäss einem der Ansprüche 17 - 25, welches die Schritte einschliesst:
    (a) Herstellung eines Rekombinationsvektors gemäss Anspruch 26 oder 27;
    (b) Bereitstellung wenigstens einer mit einem Virus der Gattung der Pockenviren infizierten Zelle;
    (c) Transfektion besagter Zelle mit besagtem Rekombinationsvektor, wobei homologe Rekombination zwischen der DNA von besagtem Pockenvirus und wenigstens einem Teil von besagter Pockenvirus DNA, welche in besagtem Rekombinationsvektor enhalten ist, stattfindet; und
    (d) Isolierung eines rekombinanten, infektiösen Pockenvirus, der in der Lage ist besagtes Fremdgen, welches pro-oder eukaryotische Polypeptide kodiert, zu exprimieren, aus besagter Zelle mittels selektiver Verfahren.

29. Das Verfahren gemäss Anspruch 28, worin besagte Pockenvirus DNA Vaccinia Virus DNA ist.

30. Lebendimpfstoffe enthaltend einen rekombinanten, infektiösen Pockenvirus gemäss einem der Ansprüche 17 - 23 und einen physiologisch verträglichen Träger.

31. Verwendung eines rekombinanten, infektiösen Pockenvirus gemäss einem der Ansprüche 17 - 25 zur vorbeugenden Immunisierung.

Fig.1a

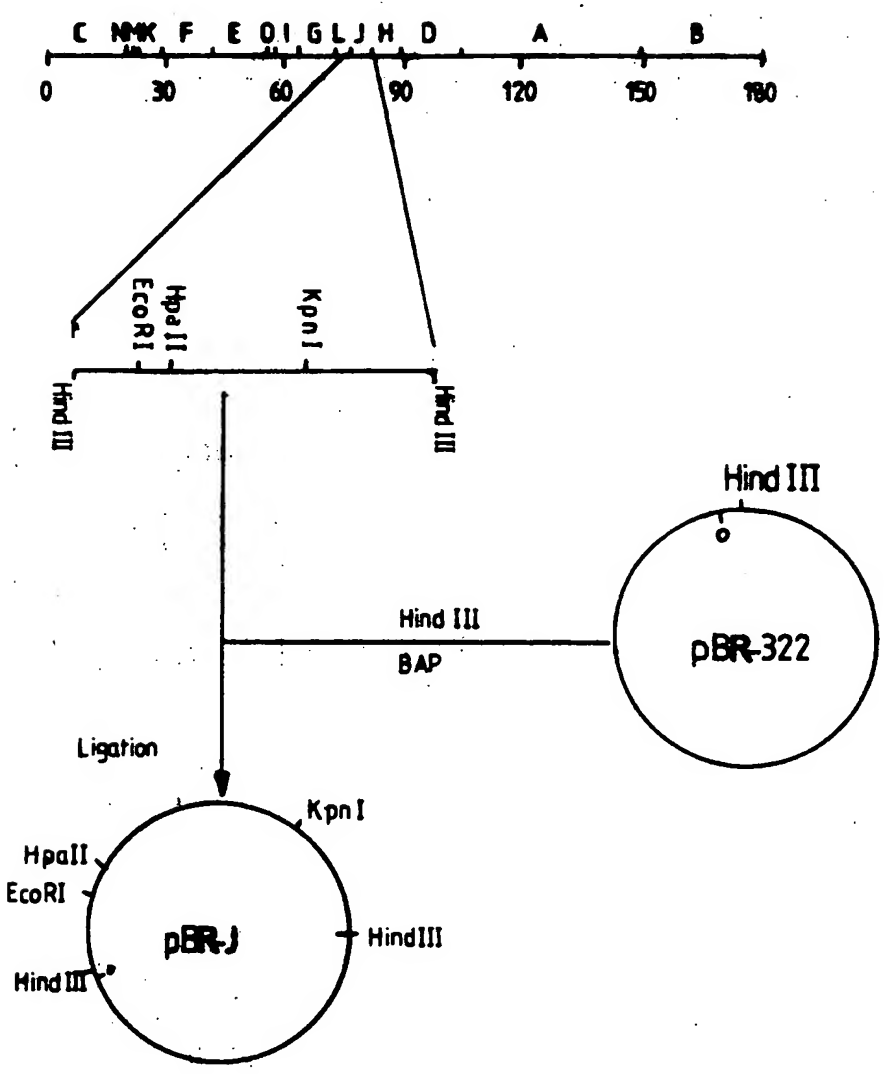

Vaccinia Hind III map
C
K
F
E
I
G
L
J
H
D
A
B
0
30
60
90
120
150
180
EcoRI
HpaII
Kpn I
Hind III
Hind III
Hind III
Hind III
BAP
pBR-322
Ligation
Kpn I
HpaII
EcoRI
pBR-J
HindIII
Hind III

Fig.1b

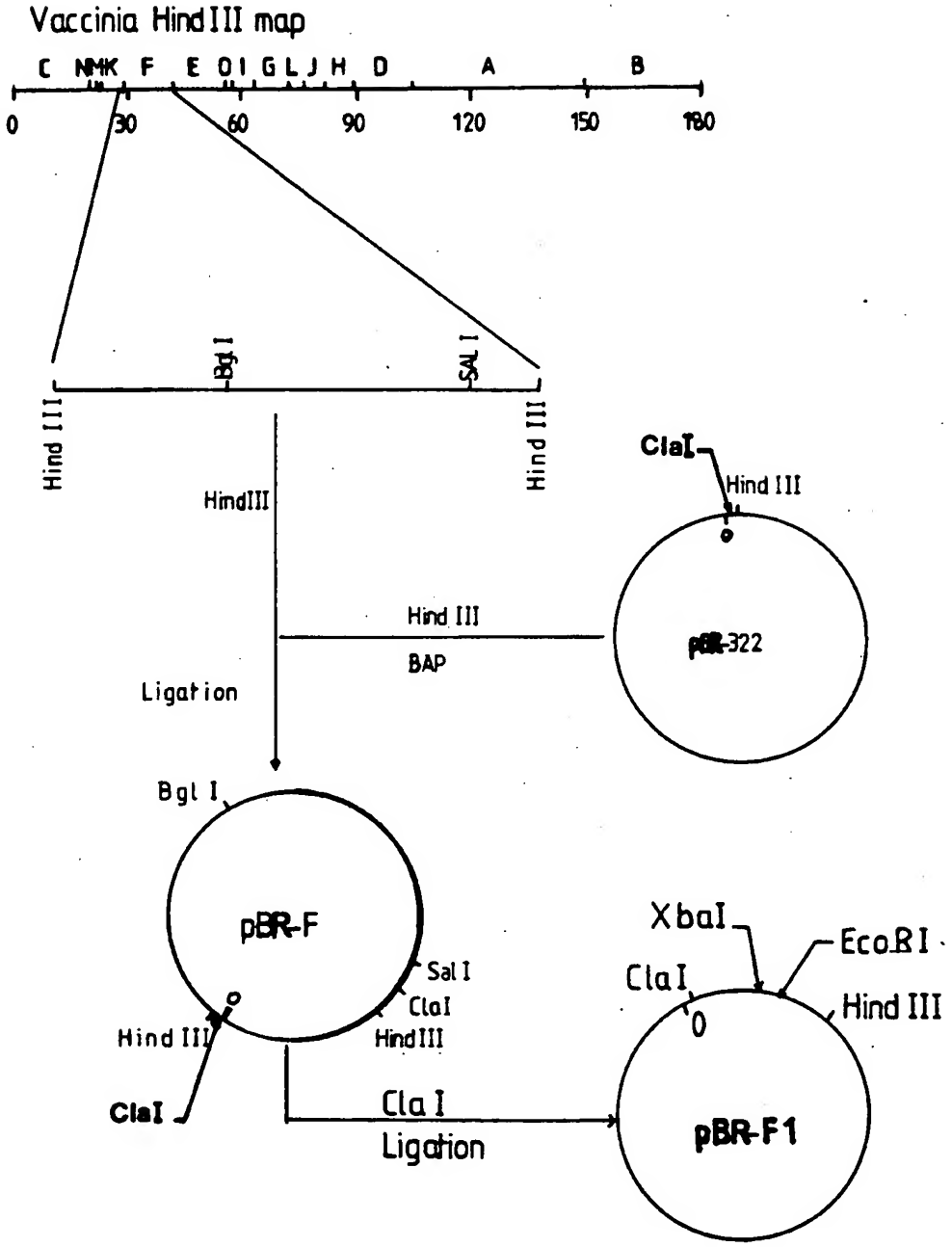

Vaccinia HindIII map
C NMK F E O I G L J H D A B
0
30
60
90
120
150
180
Bgl I
SAL I
Hind III
Hind III
HindIII
ClaI
Hind III
Hind III
BAP
pBR-322
Ligation
Bgl I
pBR-F
Sal I
ClaI
HindIII
Hind III
ClaI
Cla I
Ligation
XbaI
EcoRI
ClaI
Hind III
pBR-F1

Fig. 2

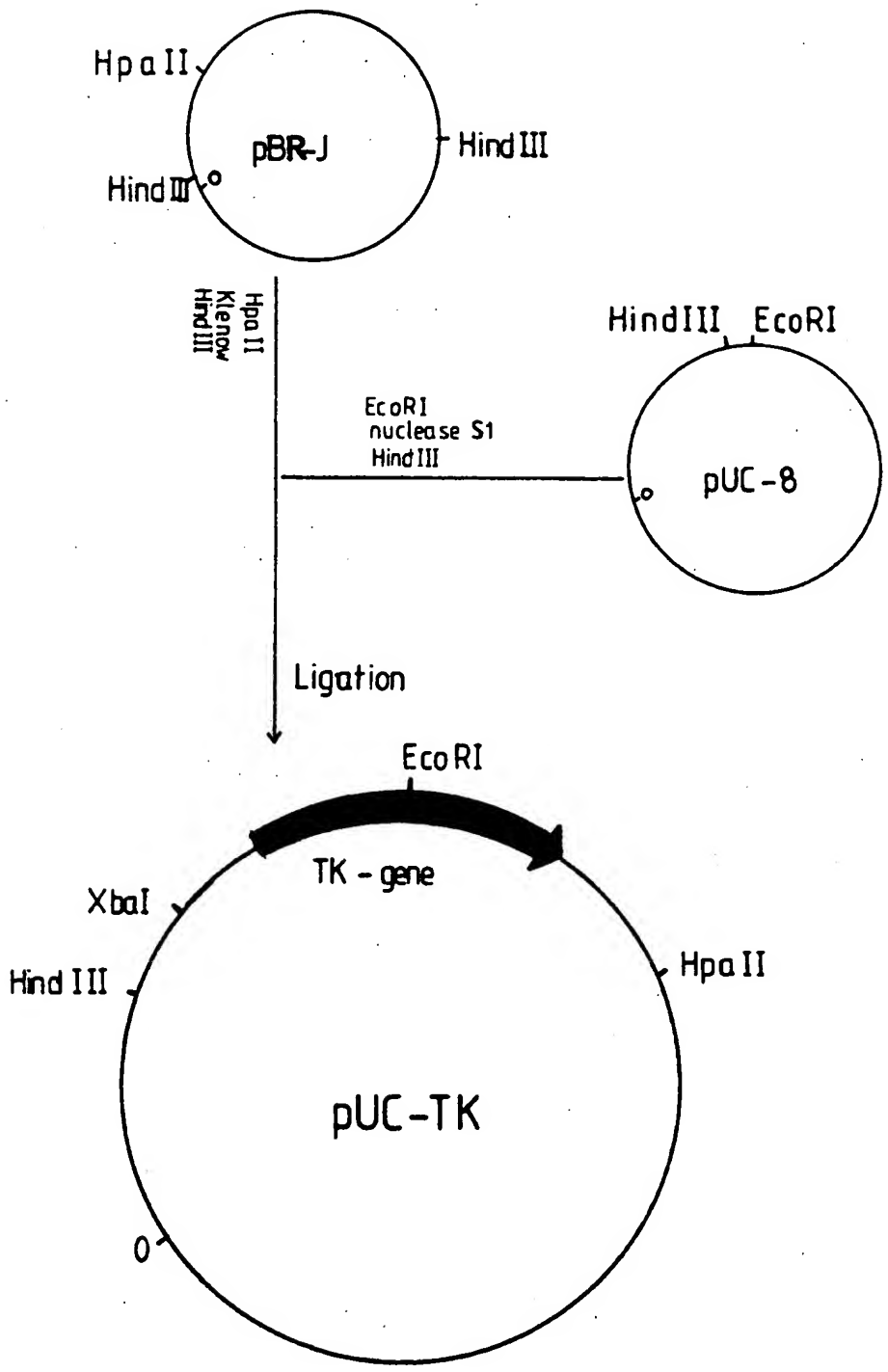

Hpa II
pBR-J
Hind III
Hind III
Hpa II
Klenow
Hind III
Hind III EcoRI
EcoRI
nuclease S1
Hind III
pUC-8
Ligation
EcoRI
TK - gene
Xba I
Hind III
Hpa II
pUC-TK
0

Fig. 3

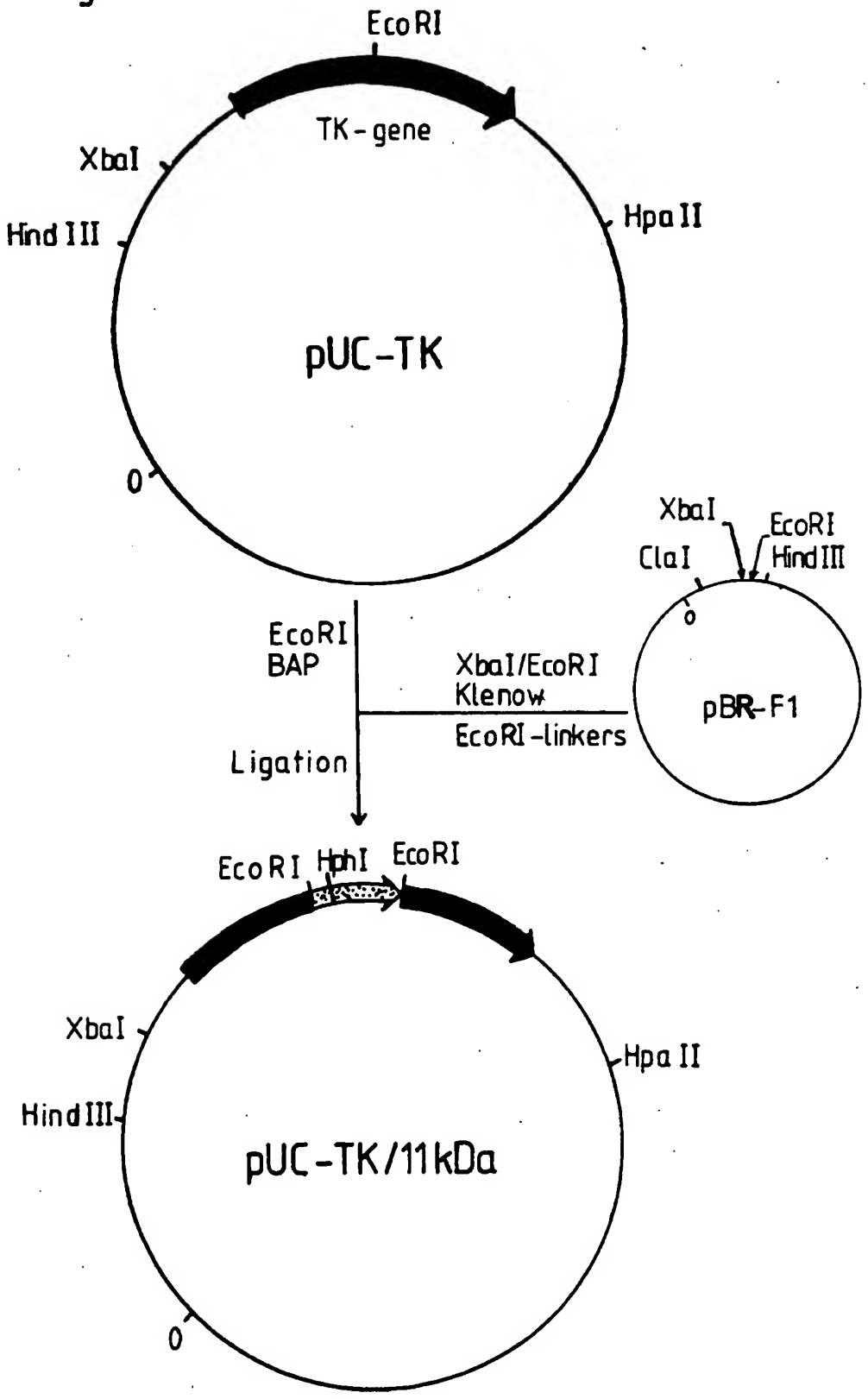

EcoRI
TK-gene
XbaI
HindIII
HpaII
pUC-TK
0
XbaI
EcoRI
ClaI
HindIII
0
EcoRI
BAP
XbaI/EcoRI
Klenow
EcoRI-linkers
pBR-F1
Ligation
EcoRI
HphI
EcoRI
XbaI
HpaII
HindIII
pUC-TK/11kDa
0

Fig. 4

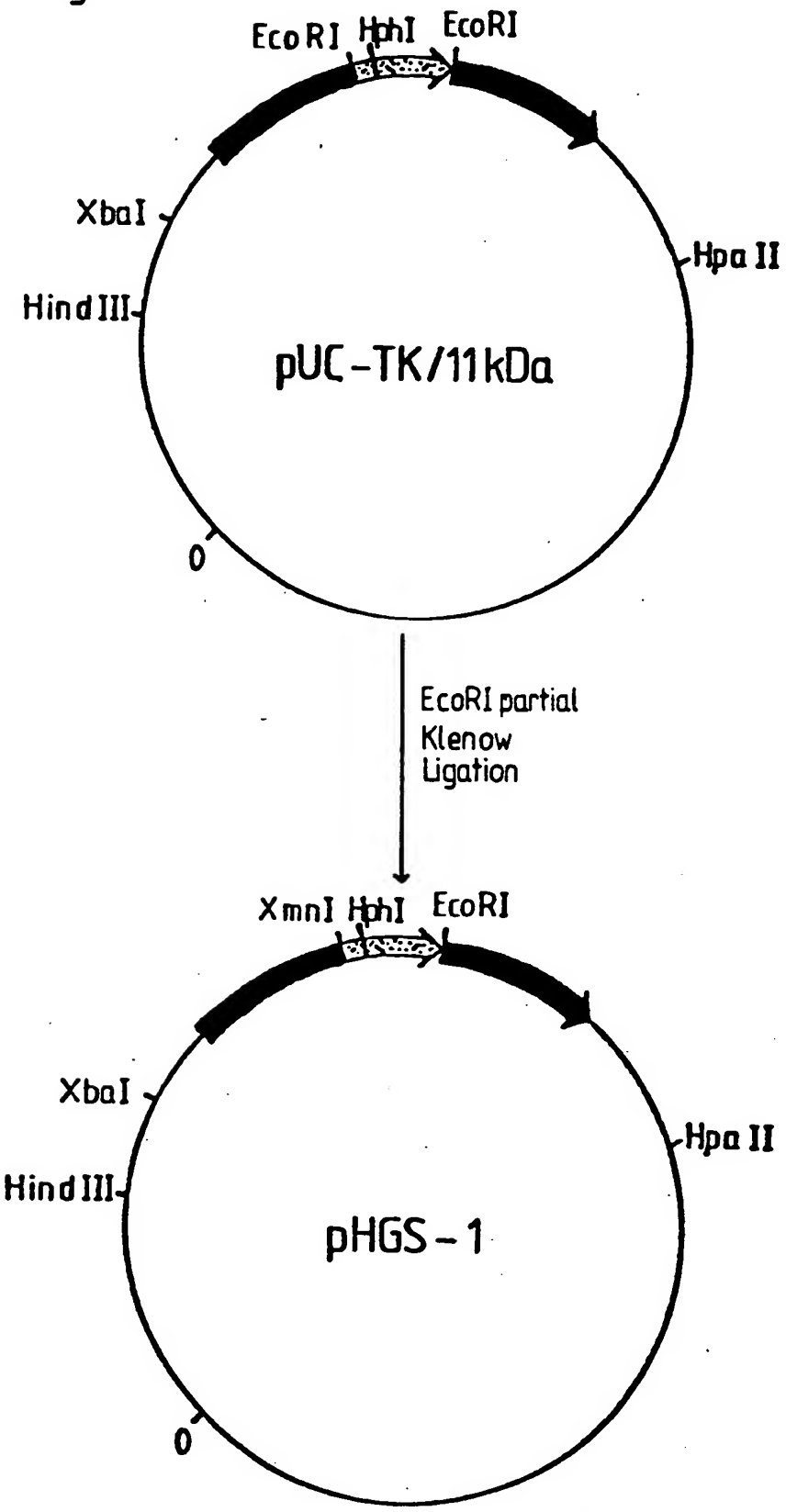

EcoRI
HphI
EcoRI
XbaI
Hpa II
Hind III
pUC-TK/11kDa
0
EcoRI partial
Klenow
Ligation
XmnI
HphI
EcoRI
XbaI
Hpa II
Hind III
pHGS-1
0

Fig. 5a

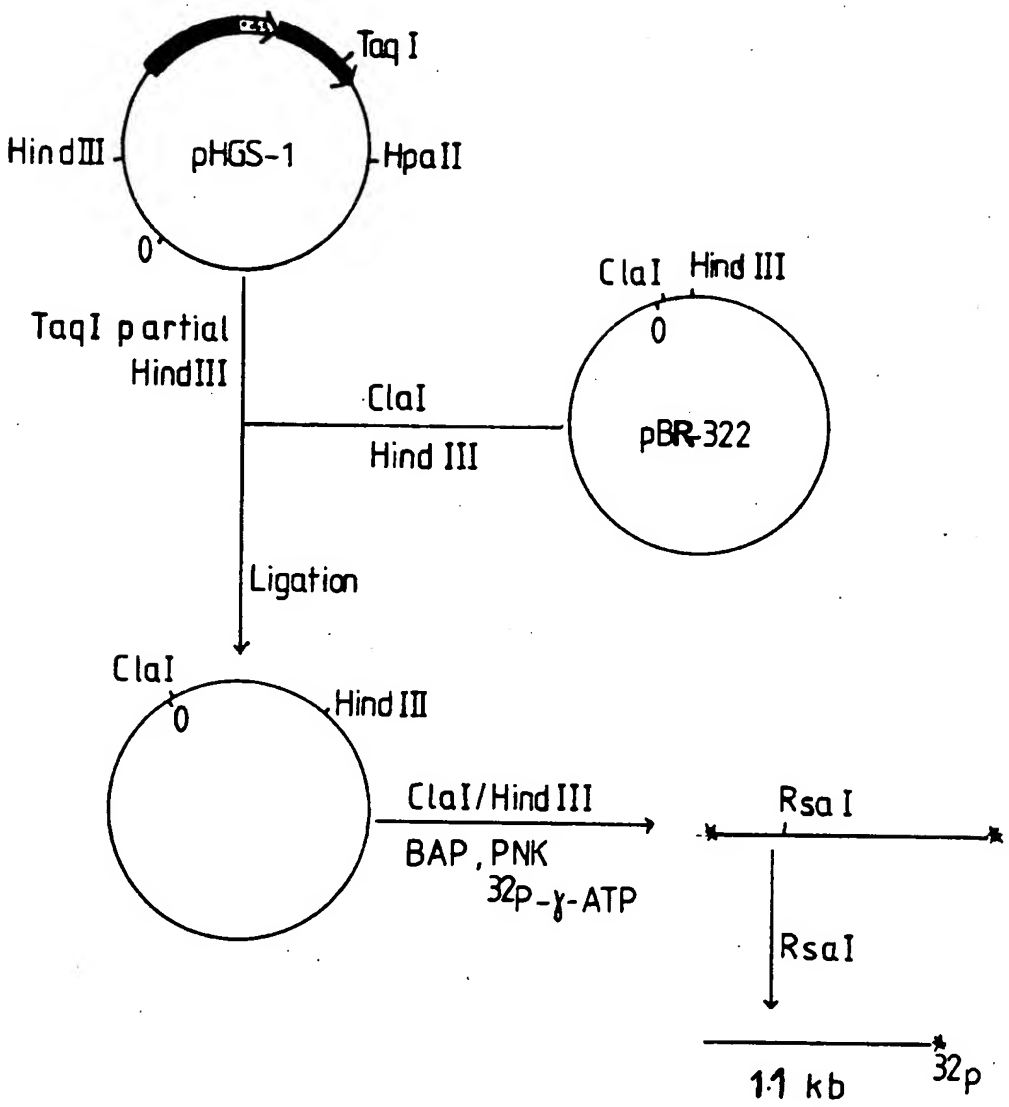

Taq I
HindIII
pHGS-1
HpaII
0
ClaI
Hind III
0
TaqI partial
HindIII
ClaI
Hind III
pBR-322
Ligation
ClaI
0
Hind III
ClaI/Hind III
BAP, PNK
32P-γ-ATP
Rsa I
RsaI
1.1 kb
32P

Fig. 5b

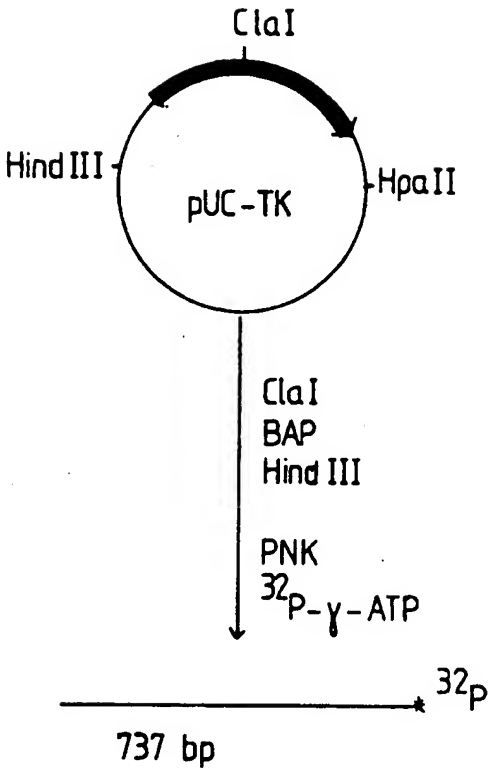

ClaI
Hind III
pUC-TK
HpaII
ClaI
BAP
Hind III
PNK
32P-γ-ATP
32P
737 bp

Fig. 6

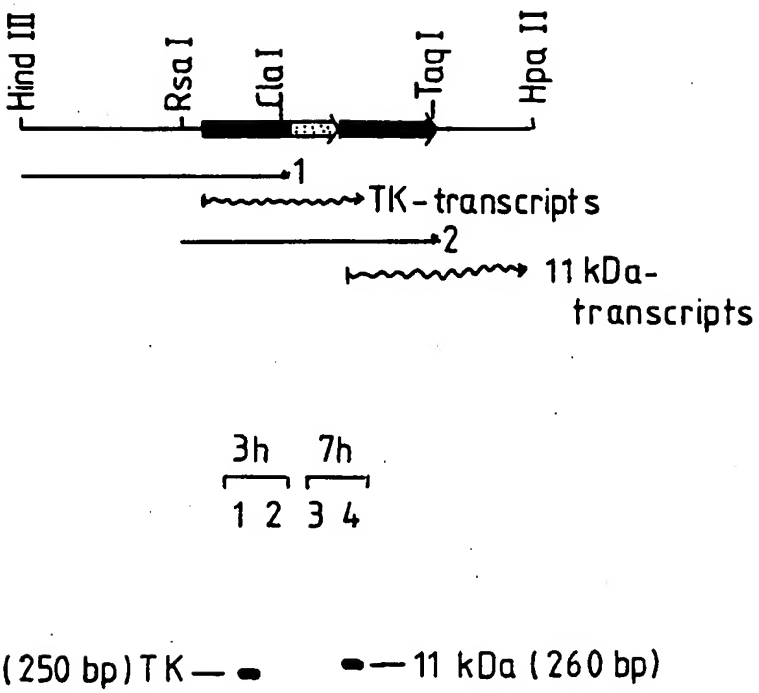

Hind III
Rsa I
Cla I
Taq I
Hpa II
1
TK-transcripts
2
11 kDa-
transcripts
3h
7h
1 2 3 4
(250 bp) TK
11 kDa (260 bp)

Fig.7

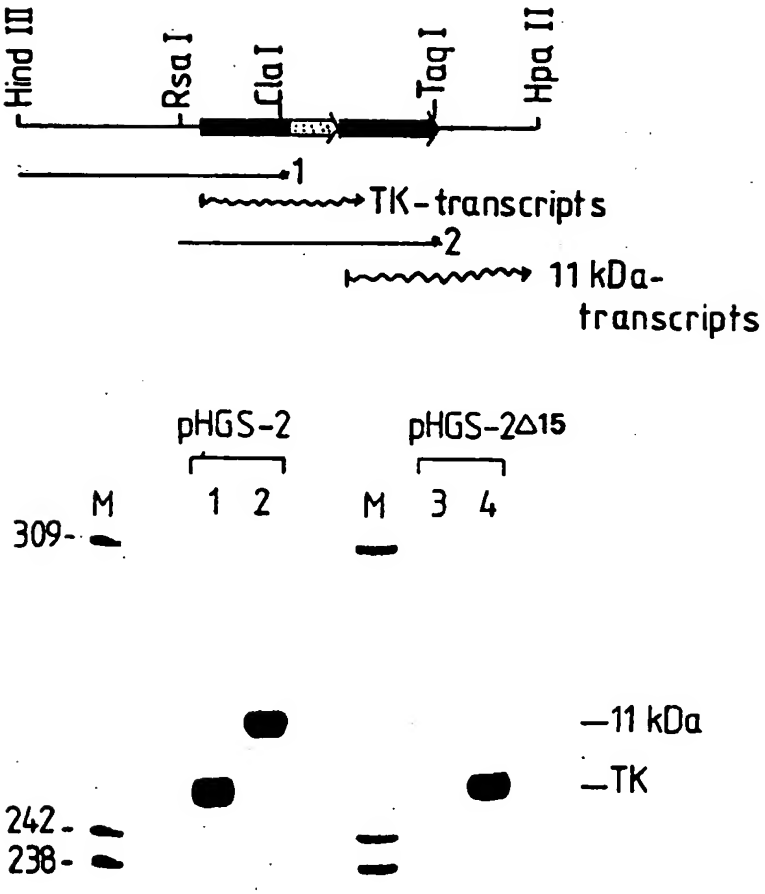

Hind III
Rsa I
Cla I
Taq I
Hpa II
1
TK-transcripts
2
11 kDa-
transcripts
pHGS-2
pHGS-2Δ15
M
1 2
M
3 4
309-
—11 kDa
—TK
242-
238-

Fig. 8

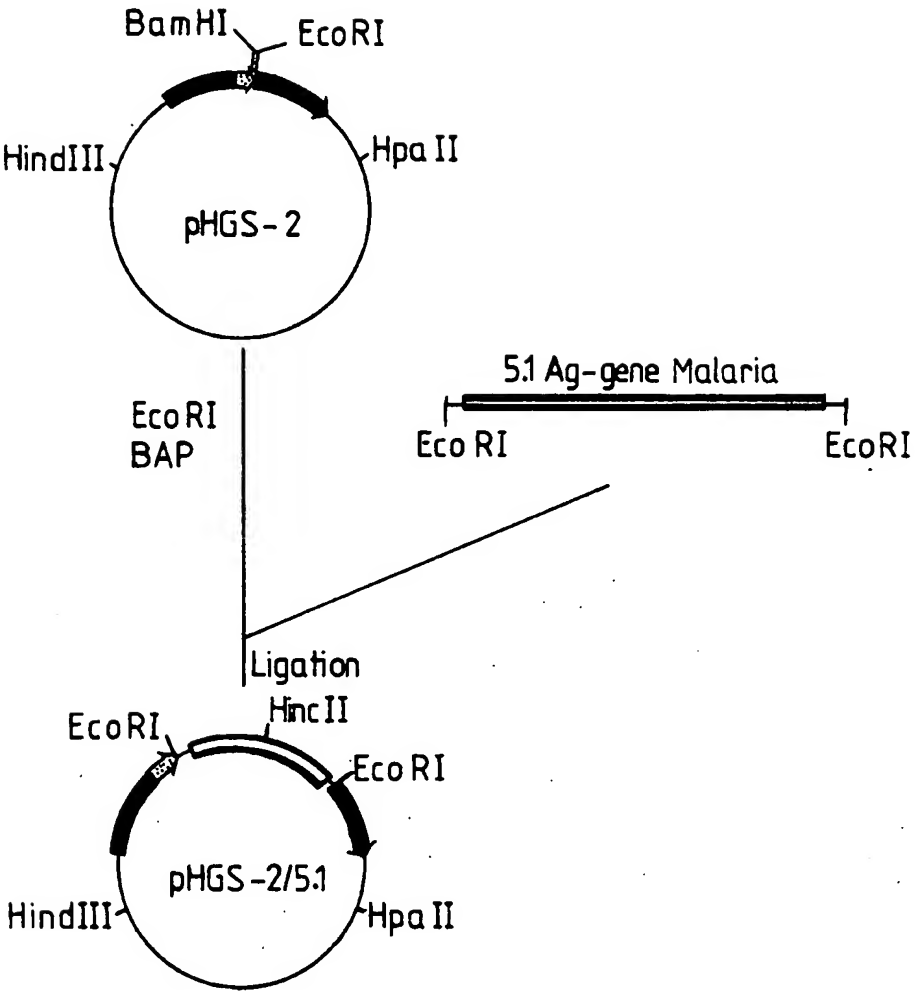
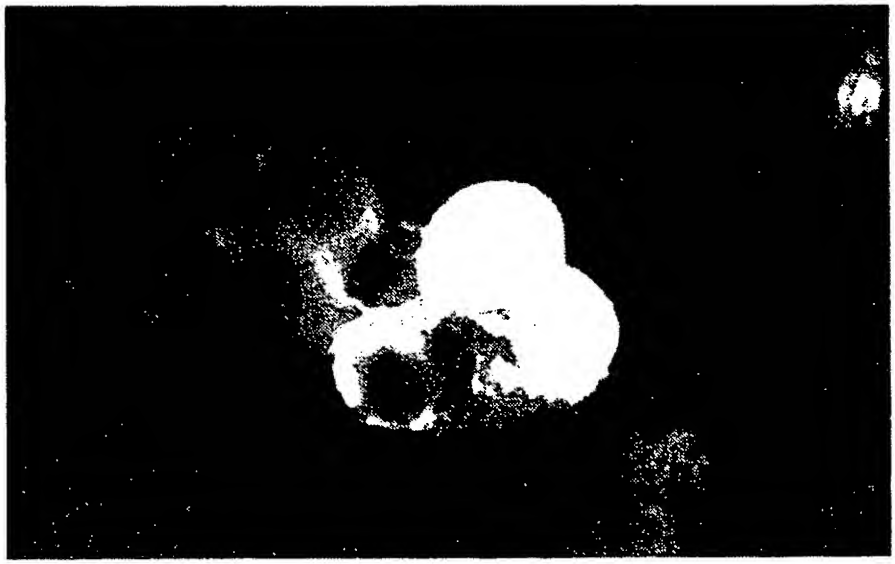

BamHI
EcoRI
HindIII
Hpa II
pHGS-2
EcoRI
BAP
5.1 Ag-gene Malaria
Eco RI
EcoRI
Ligation
EcoRI
Hinc II
Eco RI
pHGS-2/5.1
HindIII
Hpa II

Fig. 9

a



b

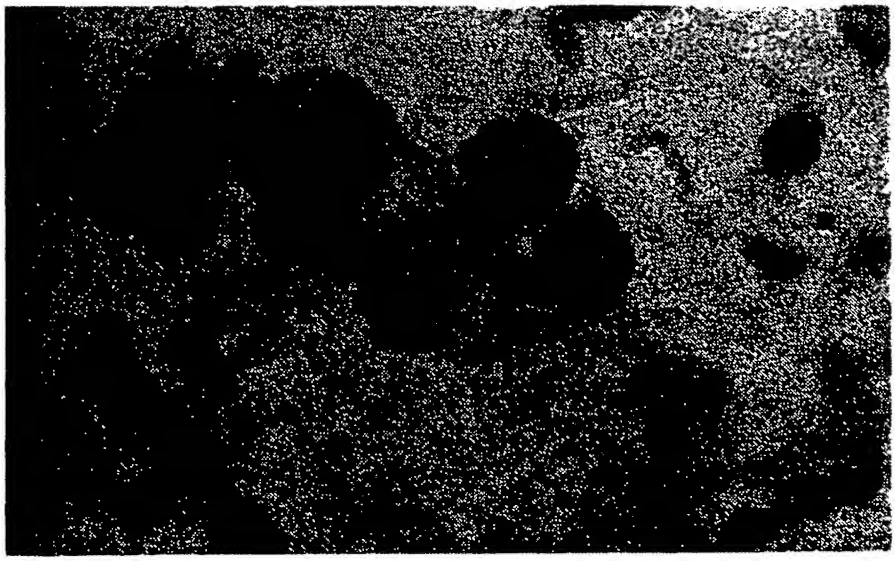


Fig. 10

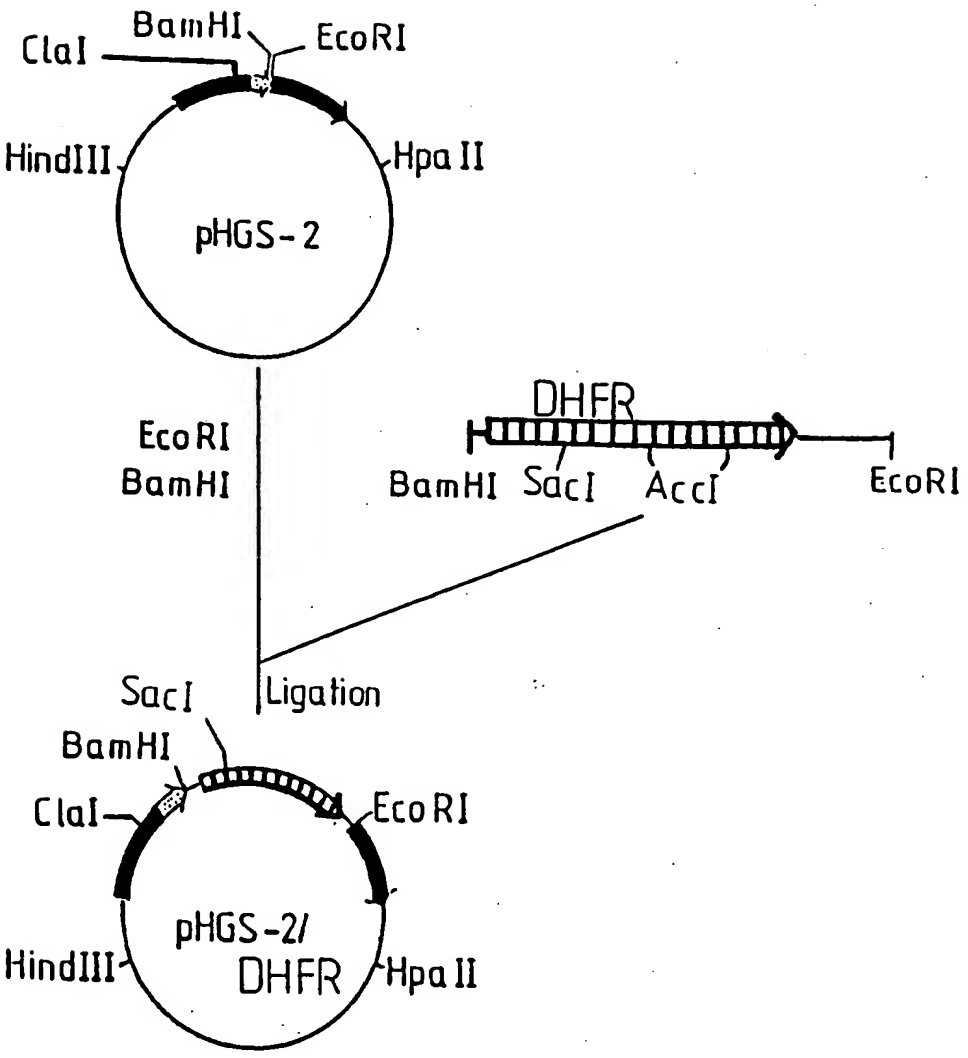

BamHI
EcoRI
ClaI
HindIII
Hpa II
pHGS-2
EcoRI
BamHI
DHFR
BamHI
SacI
AccI
EcoRI
Ligation
SacI
BamHI
ClaI
EcoRI
pHGS-21
DHFR
HindIII
Hpa II

Fig.11

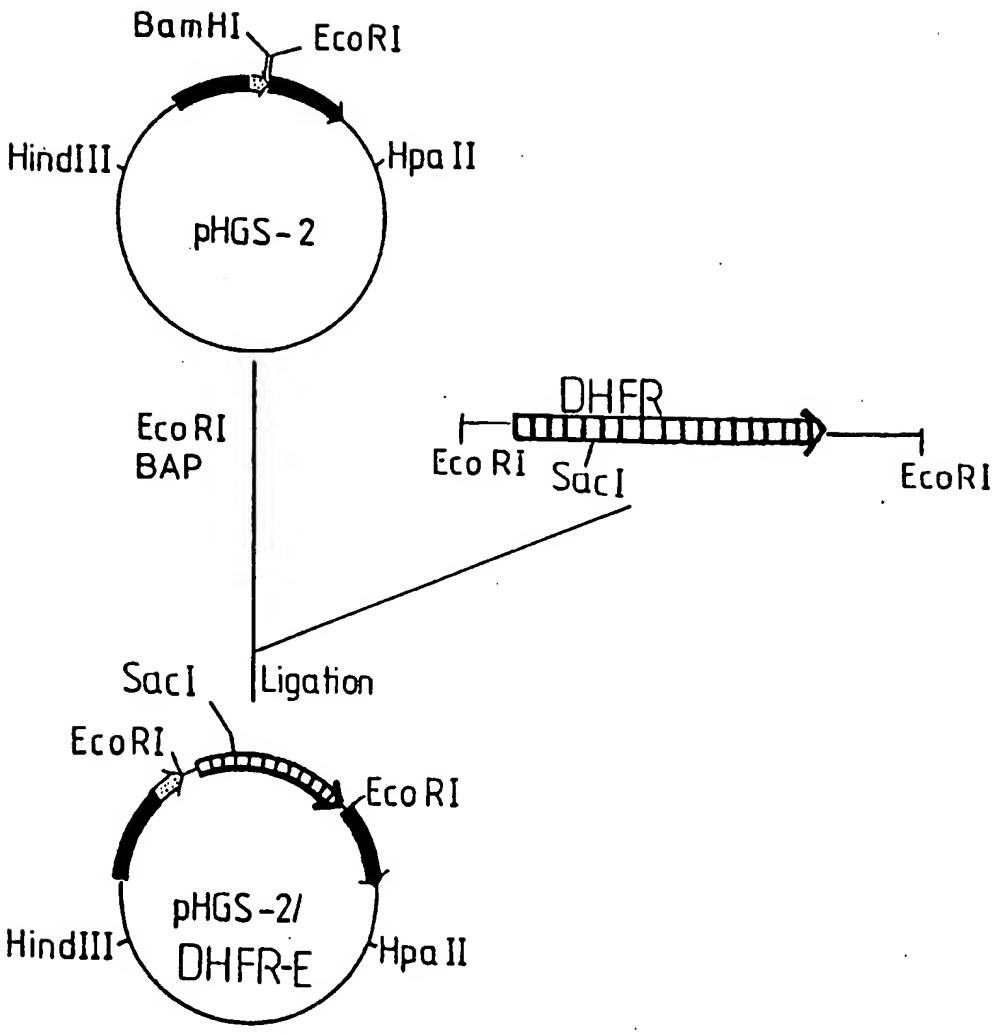

BamHI
EcoRI
HindIII
Hpa II
pHGS-2
EcoRI
BAP
DHFR
Eco RI
SacI
EcoRI
SacI
Ligation
EcoRI
Eco RI
pHGS-2/
DHFR-E
HindIII
Hpa II

Fig.12

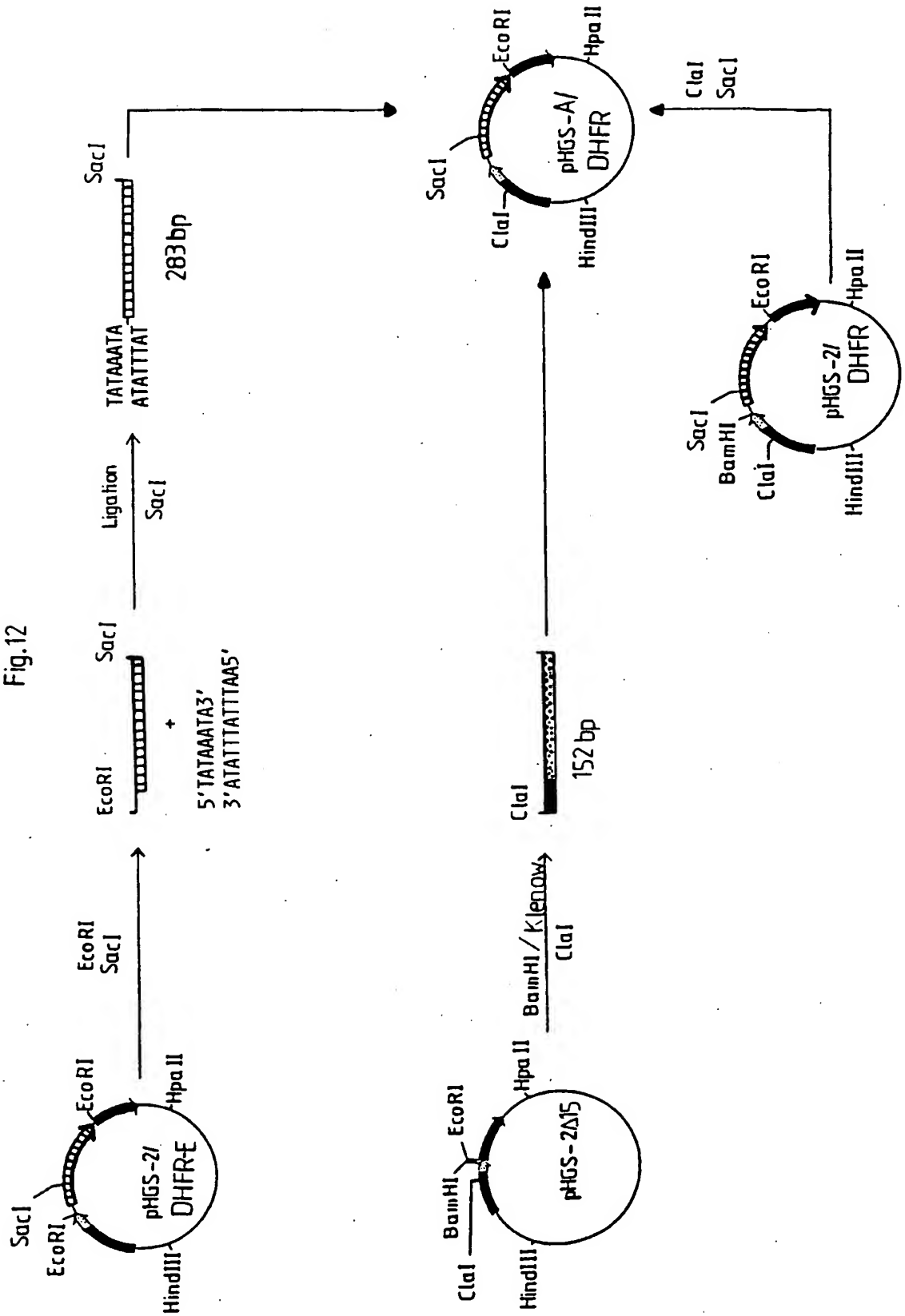

pHGS-2/ DHFR-E
Sac I
EcoRI
HindIII
EcoRI
Hpa II
EcoRI
Sac I
EcoRI
Sac I
Ligation
Sac I
TATAAATA
ATATTTAT
283 bp
5'TATAAATA3'
3'ATATTTATTTAA5'
pHGS-2Δ15
Cla I
BamHI
HindIII
EcoRI
Hpa II
BamHI/Klenow
Cla I
Cla I
152 bp
pHGS-A/ DHFR
Sac I
Cla I
HindIII
EcoRI
Hpa II
Cla I
Sac I
pHGS-2/ DHFR
Sac I
BamHI
Cla I
HindIII
EcoRI
Hpa II

Fig. 13

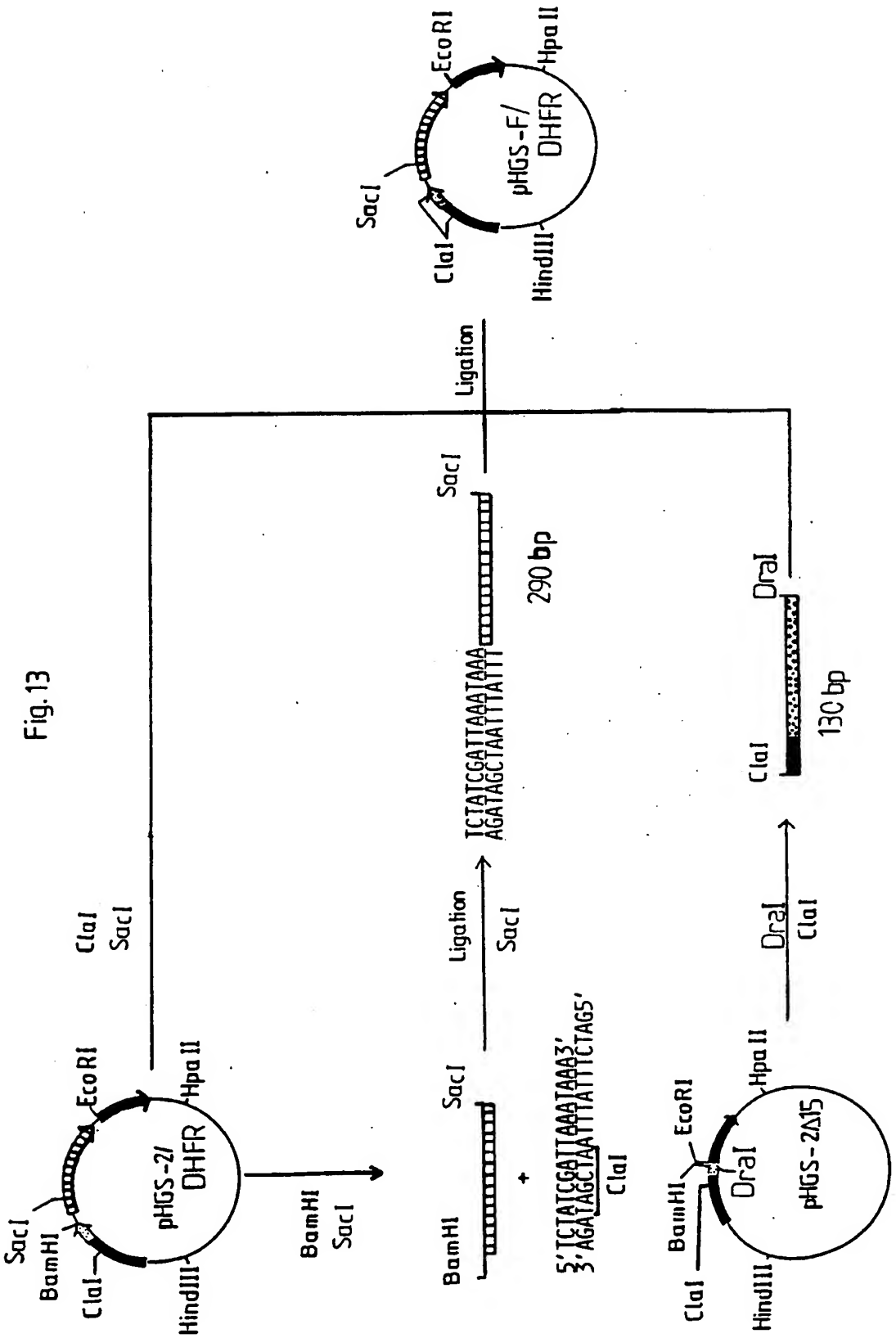
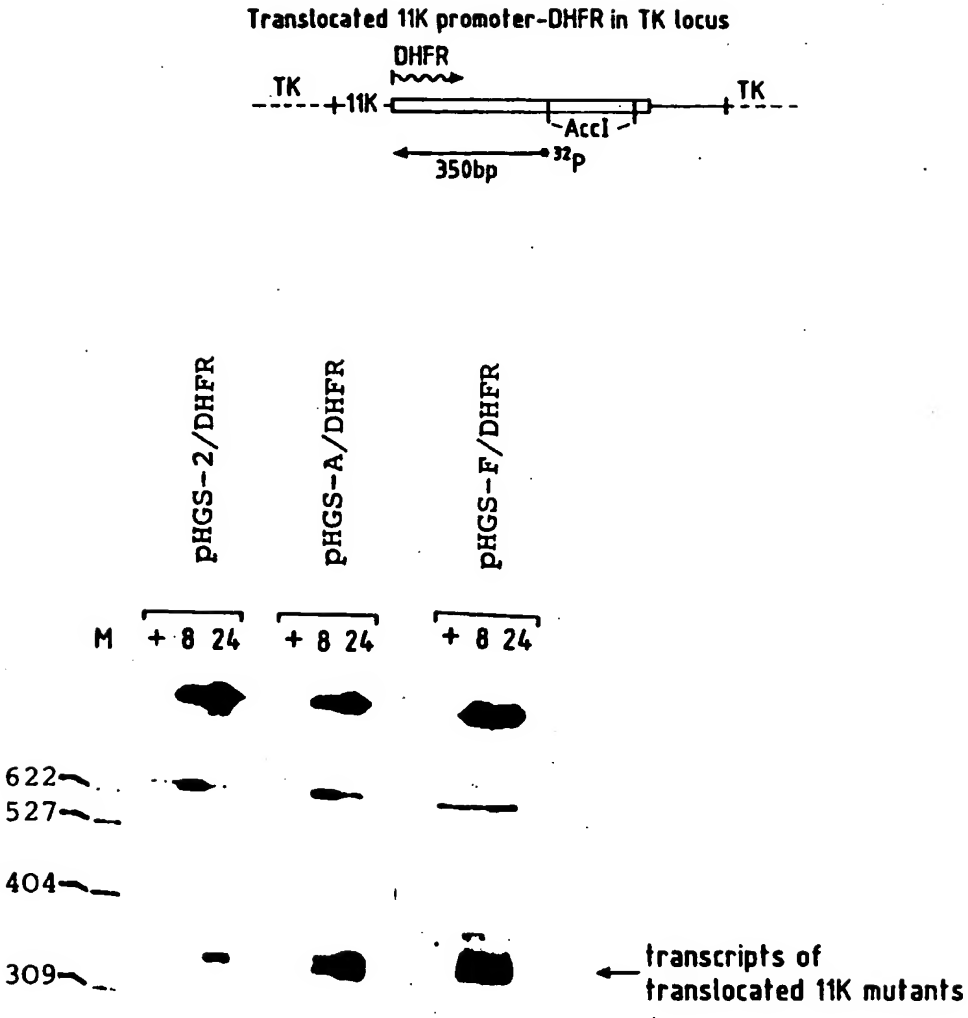

pHGS-2/ DHFR
Sac I
BamHI
Cla I
Hind III
Eco RI
Hpa II
Sac I
Cla I
BamHI
Sac I
Ligation
Sac I
5'ICTATCGATTAAATAAA
3'AGATAGCTAATTTATTT
290 bp
Cla I
Dra I
130 bp
Dra I
Cla I
pHGS-2Δ15
BamHI
Cla I
Eco RI
Dra I
Hpa II
Hind III
pHGS-F/ DHFR
Sac I
Cla I
Eco RI
Hpa II
Hind III

Fig. 14


Translocated 11K promoter-DHFR in TK locus
DHFR
TK
+11K
TK
AccI
350bp
32P
pHGS-2/DHFR
pHGS-A/DHFR
pHGS-F/DHFR
M
+ 8 24
+ 8 24
+ 8 24
622
527
404
309
transcripts of
translocated 11K mutants